PHARMACOLOGICAL PROPERTIES OF SWAZI MEDICINAL PLANTS

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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree of Master of Science in Medicine (Pharmacology).

Johannesburg, 2009.

DECLARATION

I, Gugu Fortunate Sibandze declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine (Pharmacology) at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

Signature:.....

On this……..... day of …..... 2009.

DEDICATION

This dissertation is dedicated to my husband Maqhawe and my son Lwandzile who have continuously supported me throughout the course of my studies. I would not be where I am if it were not for your love and understanding. You encouraged me when I felt like giving up.

Thank You!

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The South African National Biodiversity Institute is thanked for the use of data from the National Herbarium, Pretoria (PRE) Computerised Information System (PRECIS). Distribution maps for the species selected in the study were sourced from them.

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ABSTRACT

Traditional medicine is widely used as a source of primary healthcare for more than 80% of the population of Swaziland. Traditional healers use various plants and combinations thereof to manage different ailments. In consultation with Swazi traditional healers, fifteen Swazi plants were selected on the basis of their ethnomedical history. These plants were collected from the Manzini region, extracted with dichloromethane/methanol (1:1) and screened for their antimicrobial, antimalarial and anti-oxidant activities and toxicity profiles. Ultra performance liquid chromatography and high performance thin layer chromatography (HPTLC) was performed to determine chemical profiles of the most active plant extracts. These chromatograms revealed the complexity of each plant extract and provided a fingerprint for each species studied.

Antimicrobial activity was determined using the minimum inhibitory concentrations assay against *Staphylococcus aureus* (ATCC 25923), *S. epidermidis* (ATCC 2223), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (NCTC 9633) and *Candida albicans* (ATCC 10231). A number of the plant extracts (51.52%) displayed good antimicrobial activity with MIC values ranging from 0.0013 to 1.00 mg/ml, with *S. epidermidis* being the most susceptible. The fruit of *Ozoroa sphaerocarpa* (MIC value = 0.001 mg/ml) and the leaves of *Syzygium cordatum* (MIC value = 0.04 mg/ml) were the most active against this pathogen. The HPTLC-bioautography results showed a number of compounds within each extract, such as the fruit of *O. sphaerocarpa* and leaves of *S. cordatum*; that were responsible for the inhibitory activity against *S. epidermidis*, which correlated to the MIC results.

Breonadia salicina, *S. cordatum* and *O. sphaerocarpa* were reported to be used in combination for the traditional treatment of diarrhoea. A combination study was designed to determine their pharmacological interaction using *E. coli* as a test pathogen. A synergistic interaction was observed between *S. cordatum* and *O. sphaerocarpa*, as well as between *S. cordatum* and *B. salicina*. When the three plants were combined in a triple combination, a synergic interaction was observed, which supports the rationale by traditional healers to use these plants in combination for the treatment of diarrhoea.

The DPPH· (2,2-diphenyl-picryl-hydrazil) scavenging activity, ferrous metal chelating activity and inhibition of lipid peroxidation were correlated to the total phenolic and flavonoid content of the plant extracts. *D. cinerea* (leaves) was the most active free radical scavenger (IC_{50}: 5.89 ± 0.39 µg/ml) as compared to ascorbic acid (IC_{50}: 5.61 ± 1.13 µg/ml). The leaves of *S. cordatum* and fruit of *Z. mucronata* had the highest content of phenolics (806.10 ± 42.28 and 536.87 ± 36.76 mg GAE/g extract), respectively; whilst the bark of *Terminalia phanerophlebia*, fruit of *O. sphaerocarpa*, bark of *S. cordatum*, and bark of *Trichilia emetica* had the highest content of flavonoids in the range of 26.40 to 33.33 mg RE/g extract. An HPTLC method showed a number of compounds which were responsible for the free radical scavenging activity in each plant extract.

Antimalarial activity against the asexual stages of the 3D7 strain of *Plasmodium falciparum* was determined using the tritiated hypoxanthine incorporation assay. Whilst the toxicity profiles of the extracts were tested on human kidney epithelial cells using the tetrazolium-based MTT viability assay; as well as using the red blood cell lysis assay. Five of the extracts displayed *in vitro* antimalarial activity with IC_{50} values less than 20 µg/ml, namely *T. phanerophlebia* (leaves), *Berkheya setifera* (stem/root), *Priva meyeri* (whole plant), *T. emetica* (leaves) and *B. salicina* (bark). The five most active extracts did not cause red blood cell lysis at concentrations ten times greater than the IC_{50} values. Furthermore, these extracts were combined with quinine at different ratios and predominantly produced antagonistic interactions, highlighting the need for extreme caution when used together with clinical antimalarial drugs. The toxicity profile of the plant extracts on human kidney epithelial cells indicated that about 54.54% of the extracts were non-toxic, with IC_{50} values greater than 100 µg/ml.

In conclusion, this study has reported for the first time on the pharmacological properties of some species such as *B. setifera* and *P. meyeri*, which display the potential of Swazi flora in the search for new and novel compounds in therapeutic drug research.

CONFERENCES / PRESENTATIONS

Sibandze GF, van Zyl RL, van Vuuren SF, Viljoen AM, Amusan OOG. 2007.

Antimicrobial and toxicity studies of Swazi medicinal plants. Oral presentation at the 7th Annual Meeting of the Indigenous Plant Use Forum (IPUF), 2nd – 5th July 2007, held at the University of Johannesburg, Johannesburg, South Africa (Abstract in Appendix A1).

Sibandze GF, van Zyl RL, van Vuuren SF, Viljoen AM, Amusan OOG. 2007.

Antimicrobial and toxicity studies of Swazi medicinal plants. Oral presentation at the Faculty Research Day, 21st – 22nd August 2007, University of the Witwatersrand, Johannesburg, South Africa (Abstract in Appendix A1).

Sibandze GF, van Zyl RL, van Vuuren SF, Viljoen AM, Amusan OOG. 2007.

Pharmacological Properties of Swazi Medicinal Plants. Oral and poster presentation at the 5th Annual South African Pharmacology and Toxicology Congress, 2nd – 5th October 2007, held at the Buffelspoort Holiday Resort, Marikana, North West Province, South Africa (Abstract in Appendix A2).

Sibandze GF, van Zyl RL, van Vuuren SF, Viljoen AM, Amusan OOG. 2008.

Pharmacological Properties of Swazi Medicinal Plants. Poster presentation at the School of Therapeutic Science Research Day, August 2008, University of the Witwatersrand, Johannesburg, South Africa (Abstract in Appendix A2).

Sibandze GF, van Zyl RL. 2008. The antimalarial and toxicity studies of Swazi medicinal plants. Poster presentation at the World Conference on Medicinal and Aromatic Plants (WOCMAP) IV, 9th – 14th November 2008, Cape Town International Convention Center, Cape Town, South Africa and published in the African Journal of Traditional, Complementary and Alternative Medicines. Abstracts of the World Conference on Medicinal and Aromatic Plants, Cape Town November 2008 pp. 467 (Abstract in Appendix A3).

PUBLICATIONS

(Not generated from this study)

Amusan OOG, Sukati NA, Dlamini PS. Sibandze FG. Some Swazi phytomedicines and their constituents. African Journal of Biotechnology. 2007; 6: 267-272.

Aziba PI, Sandahl JF, Sibandze G. Antispasmodic, analgesic, and sedative effects of *Mormodica involucrate* aqueous extract in rodents. Pharmaceutical Biology 2007; 45: 339-341.

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

AIDS: acquired immune deficiency syndrome

ANOVA: analysis of variance

ATCC: American Type Culture Collection

BPI: base peak intensity

CFU: colony forming unit

CO_2: carbon dioxide

°C: degrees Celsius

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

DPPH: 2,2-diphenyl-1-picrylhydrazyl

EDTA: ethylenediaminetetraacetic acid

FIC: fractional inhibitory concentration

GPS: global positioning system

GPx: glutathione peroxidase

GSH: glutathione

HEPES: N-2-hydroxyethyl-piperazine-N'-2-ethane-sulfonic acid

HIV: human immune virus

HPLC: high-performance liquid chromatography

HPTLC: high-performance thin layer chromatography

H_2O_2: hydrogen peroxide

IC_{50}: concentration causing 50% cell death

INT: *p*-iodo-nitrotetrazolium violet

KCl: potassium chloride

KH_2PO_4: potassium dihydrogenphosphate

kv: kilovolt

M: molar

mg: milligram

MIC: minimum inhibitory concentration

ml: milliliter

MMC: mutagen mitomycin C

Ms: mass spectrometry

MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide

m/z: mass to charge ratio

µg: microgram

µl: microlitre

µM: micromolar

n: number of experimental repeats

N_2: nitrogen

NaCl: sodium chloride

NADH: nicotinamide adenine dinucleotide

NADPH: nicotinamide adenine dinucleotide phosphate

NaCl: sodium chloride

$NaHCO_3$: sodium hydrogencarbonate/sodium bicarbonate

$Na_2HPO_4.2H_2O$: di-sodium hydrogen phosphate dihydrate

NCTC: National Collection of Type Cultures

nm: nanometre

NO: nitric oxide

$NO^{\cdot}$: nitric oxide radical

$NO_2^{\cdot}$: nitrogen dioxide radical

O_2: oxygen

$O_2^{\cdot}$: superoxide radical

$OH^{\cdot}$: hydroxyl radical

PBS: phosphate buffer saline

pH: potential hydrogen

Prx: peroxiredoxins

RBC: red blood cell

Rf : retention factor

ROS: reactive oxygen species

$ROO^{\cdot}$: peroxyl radical

rpm: revolutions per minute

RPMI: Roswell Park Memorial Institute

Rt : Retention time

s.d.: standard deviation

SIRMIP: Swaziland Institute for Research in Traditional Medicine, Medicinal and Indigenous Food Plants

SOD: superoxide dismutase

spp: species

subsp.: subspecies

TLC: thin layer chromatography

TSA: Tryptone soya broth

TSB: Tryptone soya agar

UNDP: United Nations Development Program

UPLC: ultra performance liquid chromatography

UV: ultraviolet

v: volt

var: variety

v/v: volume per volume

WHO: World Health Organization

w/v: weight per volume

CHAPTER 1: GENERAL INTRODUCTION

1.1 Traditional medicine and the importance of plants

Traditional medicine is a source of primary healthcare in most societies in Africa, including Swaziland. About 70 – 80% of people in Africa consult traditional medical practitioners for healthcare (Cunningham, 1993). Plants are the oldest known source of human and livestock healthcare, and an important source of global biodiversity. They produce a diverse range of bio-active molecules, making them a rich source of different types of medicines (van Wyk *et al*., 1997). An estimated 50,000 plant species (most being higher plants) are used in traditional medicine, especially in developing countries where access to modern healthcare is limited by various factors, including the cost of importing modern drugs and the remoteness of hospitals and clinics from most of those who need them (UNDP, 2005). In such cases, the traditional healers are often the only source of healthcare service, and in many cases, the preferred source.

According to Samuelsson (1999), traditional medicine has provided western medicine with more than 40% of all pharmaceuticals such as the malaria drugs artemisinin and quinine. The use of medicinal plants as a source for relief from illness is an ancient practice evidenced by the early Chinese and Indian manuscripts (Hamburger and Hostettmann, 1991). Traditional or indigenous systems of medicines have persisted for many centuries even where modern healthcare is readily available (Kaido *et al*., 1997).

The revival of interest in the use and importance of African medicinal plants by the World Health Organisation (WHO) and many developing countries has led to the intensified efforts on the documentation of ethnobotanical data of medicinal plants, since most traditional healers keep no records and their information is passed on by word of mouth from generation to generation (Sofowora, 1993). Traditional medicines are used to treat numerous ailments including diarrhoea, nausea, infections, malaria and cancer.

1.2 Traditional medicine in Swaziland

At least 85% of the Swazi population make use of the traditional system of medicine for their primary healthcare. Traditional healing is linked to the cultural beliefs of the Swazi people as well as their belief about the cause of illness; and it is believed to satisfy the physical, mental and spiritual needs of those who participate in the system (Green and

Makhubu, 1984). Swazi traditional healing is based on a combination of naturally-derived medicines more than rituals. These medicines are both from plant and animal origin and are prepared in various ways, ranging from boiling to burning (Green and Makhubu, 1984). Plants are of great importance in traditional medicine as they form the major ingredient in the preparation of remedies. Plant parts that are used range from the fruits to the roots, whilst animal parts may be animal fat or body parts. They are administered orally, as enemas, as poultices, by inhalation, as purgatives and snuff. For most Swazis, health problems fall into three categories: those best treated by doctors, those best treated by traditional healers and those that fall in between where either type of treatment – or combination of the two – may be effective.

1.2.1 Types of traditional healers in Swaziland

There are basically three types of traditional practitioners in Swaziland, the diviner-healer (*inyanga/sangoma*), the herbalist (*lugedla, inyanga yemitsi*) and the faith healer (*umprofethi).* The diviner-healer works with spirits who are said to help in diagnosis and healing of the disease. The spirit may either be an ancestral one (*emadloti*) or a foreign one (*emanzawe, tinzunza* or *benguni*) (Gort, 1989). The herbalists differ from the diviner in the sense that they do not divine but, make a diagnosis on the basis of physical and mental symptoms. Knowledge about the use of herbs might have been passed on from a family member or simply because they had an interest about the use of herbs and went through a training process. The faith healer is different from the latter two healers, in that they are affiliated to an organised church; however, they share the same theory of health and disease as the *inyanga* or *sangoma*. They divine in a manner that is similar to that of diviner-healers, but instead of consulting spirits, they claim to hear from God and angels (Green and Makhubu, 1984).

1.2.2 Ratio of healers to people

Traditional healers far outnumber modern health care practitioners in Swaziland and are located in the communities where the people are. This results in preference by the people to consult traditional healers as they are also cheaper and need not only be paid using money, which is scarce in the rural areas. The ratio of practitioners to population in Swaziland is 1:100 for the traditional healer and 1:10 000 for the modern medical doctor (University of Swaziland, 1999). These figures show the need for incorporation of the traditional healers into the National healthcare system to facilitate their monitoring. The frequency of consultation and traditional medicine use also emphasises the need for

scientific research into herbal remedies to prove the efficacy of the traditional systems of medicine.

1.2.3 Integration of traditional medicine into Swazi healthcare system

In Swaziland, there are two traditional healers' organizations; the Traditional Healers Organization and the Tinyanga Tendzabuko Organization. Traditional healers are affiliated to either of these organizations, however, there are others who are not affiliated to any of the organizations, meaning that they are not monitored or accountable to anybody and can exploit the public without having to answer to anyone (Amusan, 2007). The Ministry of Health and Social Welfare in Swaziland with the aid of the World Health Organisation is currently making efforts to integrate traditional medical practitioners into the country's health care system. This is to ensure harmonization of healthcare delivery, ensure regulation and monitoring of the practice since traditional healers will have to be registered before they can practice. However, the integration of traditional healers into the healthcare system is faced with numerous challenges which include mainly the safety and efficacy of the traditional medicines, the diagnosis of the diseases and training of healers. Traditional healers propose collaboration on equal terms with orthodox medical practitioners rather than integration (Green and Makhubu, 1984; Amusan, 2007).

1.3 Some challenges facing traditional medicine globally

Though traditional medicine is widely practiced, it is still faced with a number of challenges that need to be addressed to facilitate its integration into national healthcare systems. Firstly, most countries (Swaziland included) do not have a national policy for traditional medicine. Secondly, many consumers that use traditional medicine have the misconception that "natural" means "safe" and may not be aware of their potential toxicity and adverse effects. This is also exacerbated by the fact that most countries have no monitoring systems when it comes to herbal preparations. Thirdly, the sustainable use of natural resources in traditional medicine is a cause for concern. Nowadays, traditional medicine has been commercialised and harvesting in large quantities, especially of plant material by traders may lead to extinction of some plant species (Figure 1.1). Lastly, the lack of appropriate training for providers and proper qualification and licensing schemes make it difficult for national authorities and consumers to identity qualified traditional medicine providers (WHO, 2003).

Figure 1.1: Faraday Muti Market in Johannesburg, South Africa. Unsustainable harvesting of large quantities of plant bark material (Photographer: RL van Zyl).

1.4 Medicinal plant-drug interactions

A number of plant drug interactions have been reported in literature. Examples include the concomitant use of Ginkgo (*Ginkgo biloba*), ginseng (*Panax* spp) or garlic (*Allium sativum*) with warfarin and antiplatelet drugs (Valli and Giardina, 2002). St John's wort (*Hypericum perforatum*) with its main constituents being hypericin, hyperforin and flavonoids, is traditionally used to treat inflammatory conditions as well as depression. St John's wort is contraindicated with many pharmaceutical drugs such as digoxin, theophylline, oral contraceptives, warfarin, nevirapine and cyclosporine (Bhattaram *et al*., 2002; Valli and Giardina, 2002; Evans 2002; Dennehy and Tsourounis, 2007). This is because St John's wort induces the human cytochrome P450 enzymes and the P-glycoprotein drug efflux pump (Constable *et al*., 2006; Skalli *et al*., 2007), resulting in the drugs being metabolized more quickly or pumped out of the target cell. This effectively means that the plasma concentration of these drugs is reduced and the therapeutic concentrations are not reached.

Other examples of herb-drug interactions include kava kava (*Piper methysticum*) which is contraindicated when taking cimetidine and benzodiazepines, as well as feverfew (*Tanacetum parthenium*) with warfarin. Mango (*Mangifera indica*) and papaya (*Carica papaya*) are also contraindicated with warfarin as they increase the anticoagulant effect of warfarin. *Momordica charantia* (*inkakha* or bitter melon) is commonly used by black

Africans as a green vegetable, as well as to treat various ailments such as high blood pressure and diabetes; but is contraindicated when taken concurrently with anti-diabetes mellitus drugs. Garlic, ginkgo, Echinacea (*Echinacea pupurea*), ginseng, St John's wort and kava kava have all been reported to potentially interact with anticancer drugs (Skalli *et al*., 2007).

1.5 Medicinal plant research in Southern Africa

The organisation of African Unity's Scientific Technical and Research Commission has been a spearhead in the research into African traditional medicine through funding ethnobotanical surveys of the region (Sofowora, 1996). Since then, there have been many different bodies that have embarked on ethnomedicinal research. The ethnobotanical survey route has been identified as a major route in the research for plants that can provide new drugs or act as sources of an active compound. This approach has contributed to about 74% of all pharmaceutical drugs derived from plants (Farnsworth *et al*., 1985).

Since the nineties, there has been a surge of interest in South African medicinal plant research in the search for new pharmaceuticals. This interest is directly linked to the rich botanical biodiversity of the country (van Staden, 2008). Since then, there have been numerous publications reporting on the activity of various plant species and the ailments they are used to treat. The limited availability of funding to research this indigenous natural resource has resulted in relatively little scientific research being conducted. This study was therefore undertaken to tap into and better understand this under researched or completely uninvestigated biodiversity of the southern African region

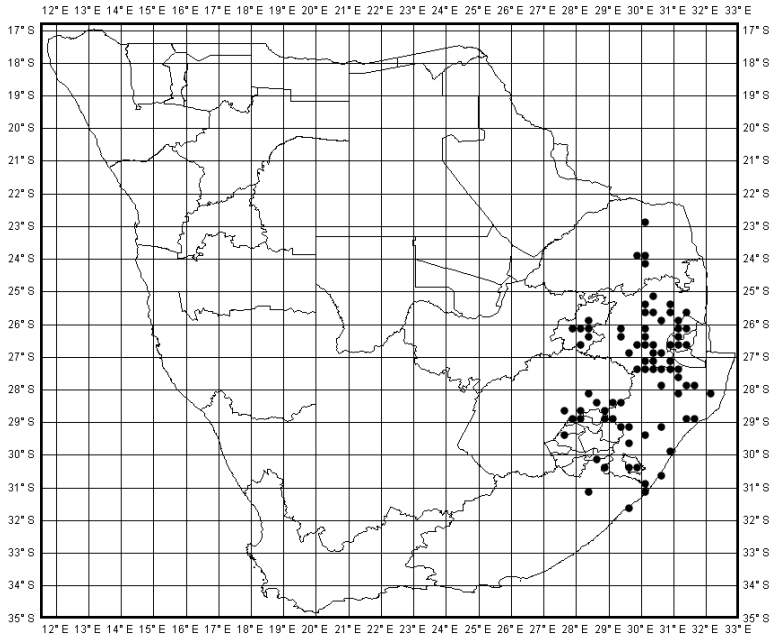
1.6 Selection of plant species studied

The selection of the plant species in this study was done based on the ethnomedical history of the plants, that is, their reported medicinal use. Preference was made to previously unnivestigated plants and especially those used to treat infectious diseases.

1.6.1 *Berkheya setifera* DC.

Berkheya setifera, also called *lulwimi lwenkhomo* in siSwati (Adeniji *et al*., 2000) is a perennial herb in the Asteraceae family with leaves that are alternate and spiny. The capitulum is heterogamous and radiate or homogamous and discoid. The phyllaries are in several series, connate at the base only, always spiny, but spines may be slender and bristle-like. The ray florets are yellow (Figure 1.2a). The plant has a wide distribution in

(a)



(b)

Figure 1.2: *Berkheya setifera* DC flower (a) (Photographer: GF Sibandze) and distribution map (b) (The South African National Biodiversity Institute).

the north-easterly part of southern Africa (Figure 1.2b) (Adeniji *et al*., 2000). Medicinally, the roots are mixed with urine and used as eardrops in the treatment of otitis media. Together with *Dicoma anomala*, the root infusion is used for toothache, sterility, jaundice, biliousness, urinary problems, burns, boils, skin conditions and abdominal disorders (Watt and Breyer-Brandwijk, 1962; Amusan *et al*., 2002; Adeniji *et al*., 2000). Another species in the genus, *Berkheya speciosa*, is used for the treatment of schistosomiasis (Sparg *et al*., 2000).

1.6.2 *Breonadia salicina* (Vahl) Hepper & J.R.I. Wood

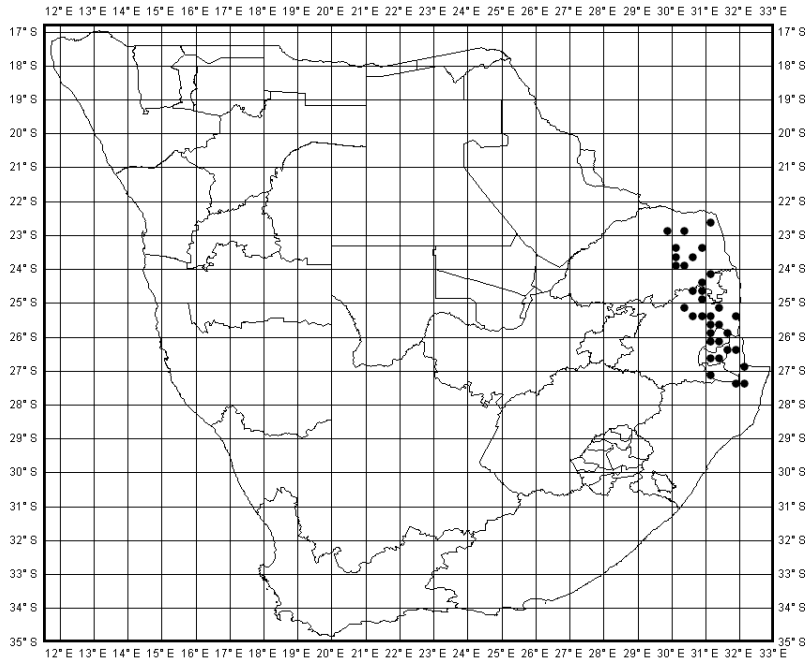
Breonadia salicina, redwood or *umhlume* in siSwati (Adeniji *et al*., 2000), is a savannah streamside tree from the Rubiaceae family and grows up 20 m in height with a dense irregular crown. The leaves are narrow and arranged in whorls at the end of the branchlets (Figure 1.3a). From December to March, the flower heads appear as long-stalked creamy,

yellowish, pinkish, pale mauve, sweet smelling globular heads and the fruit is composed of numerous small 2-celled fruitlets in a head of 6 mm in diameter. The plant is widely distributed from Tanzania in the north to KwaZulu-Natal in the south (Figure 1.3b). It is mostly found on stream banks in savannahs (Neuwinger, 1996; Venter and Venter, 2002). The wood is used for boat-building, furniture and beams, while the bark and roots are ingredients of a hunting poison in north Nigeria. Medicinally, a decoction of the bark is used to treat diarrhoea, bloody stool and colic, and the bark powder is sprinkled on injuries or wounds (Neuwinger, 1996; Venter and Venter, 2002; Amusan *et al*., 2005). Alkaloids have been isolated from the twigs and leaves; triterpenes and saponins are found in stems and leaves and the wood contains polyphenols and quinines; whilst the stem bark is rich in tannins (Neuwinger, 1996).

1.6.3 *Dichrostachys cinerea* (L.) Wight & Arn.

Dichrostachys cinerea, sickle bush or *lusekwane* in siSwati (PP Ndlovu; personal communication) is a semi-deciduous to deciduous tree in the Mimosaceae family and grows up to 7 m tall with an open crown (Figure 1.4a). The leaves are twice-compound ending in two leaflets with 20-27 pairs of leaflets each. The flowers appear from October to February and are a pendulous 40-50 mm long, two-coloured spikes, with the upper part being pink and lower part yellow. The shrub has a wide distribution from Ethopia to KwaZulu-Natal in the south (Figure 1.4b). It predominantly grows in woodland, forest margins and grassland (Venter and Venter, 2002). The bark is used to treat elephantiasis which is caused by a parasitic helmintic infection; while the root is used for toothache, stomach troubles, indigestion/diarrhoea, rheumatism and chest complaints. The burnt twigs or roots are used for bone fractures and sexually transmitted diseases and the leaf infusion for skin infections, postpartum pain, bronchitis, abdominal pains, epilepsy and snake bites (Watt and Breyer-Brandwijk, 1962; Eisa *et al*., 2000; Kambizi and Afolayan, 2001; Venter and Venter, 2002; Amusan *et al*., 2005; Tetali *et al*., 2009). The aerial parts of *D. cinerea* contain hentriacontanol, β-amyrin and sitosterol. The bark contains friedelin, α-amyrin and sitosterol, whilst the heartwood contains octacosanol (Joshi and Sharma, 1974).

(a)



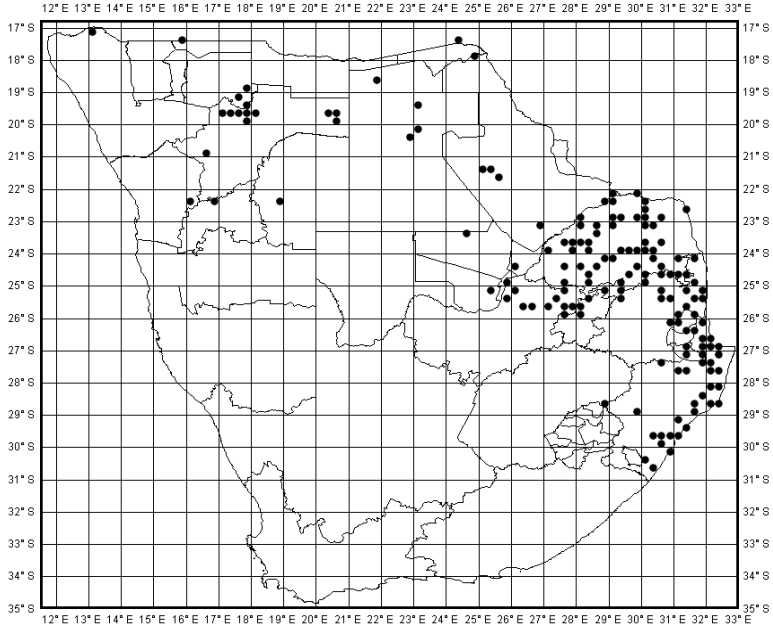
(b)

Figure 1.3: *Breonadia salicina* (a) (Photographer: GF Sibandze) and distribution map (b) (The South African National Biodiversity Institute).

1.6.4 *Ficus glumosa* Delile [syn. *Ficus sonderi*]

Ficus glumosa, synonym, *F. sonderi*, mountain fig tree or *umkhiwa* (siSwati) from the family Moraceae is a moderate sized erect tree growing up to 15 m in height, densely leafed and usually evergreen (Figure 1.5a). All parts of the tree produce a non-toxic milky latex. Figs are numerous among the leaf axils and stem and spherical, with an indefinite fruiting period (Venter and Venter, 2002). The plant is distributed from Tanzania to KwaZulu-Natal (Figure 1.5b) and found along drainage lines, rocky outcrops and cliffs in woodland and wooded grassland (Venter and Venter, 2002). The bark concoction is drunk for the treatment of diarrhoea and generalised malaise; while the milky latex is used to treat toothache, cuts, infections and sore eyes (Adeniji *et al.*, 2000; Venter and Venter, 2002; Amusan *et al.*, 2005; de Boer *et al.*, 2005).

(a)



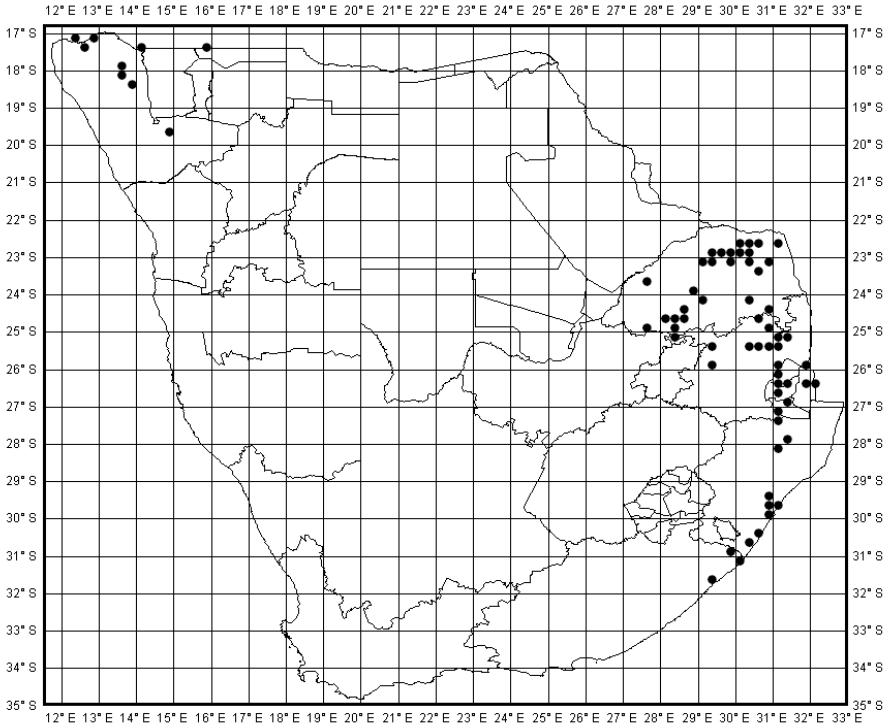
(b)

Figure 1.4: *Dichrostachys cinerea* (a) (Photographer: GF Sibandze) and distribution map (b) (The South African National Biodiversity Institute).

1.6.5 *Gardenia volkensii* K. Schum subsp. *spatulifolia*

Gardenia spatulifolia, bushveld gardenia or *manyongwana* (siSwati), from Rubiaceae, is a semi-deciduous to evergreen tree, depending on habitat (Figure 1.6a). It grows up to 8 m tall and has a roundish much branched crown. The leaves are in whorls of 3, crowded at tip of short stout branchlets and mostly spathe-shaped, glossy with an entire margin, midrib and secondary veins conspicuous on upper surface. The flower, appearing in July to December, is a single calyx ribbed with a short slit down one side and the corolla white and fragrant, turning yellow with age. The fruit is ovoid to spherical, shallowly ribbed,

(a)

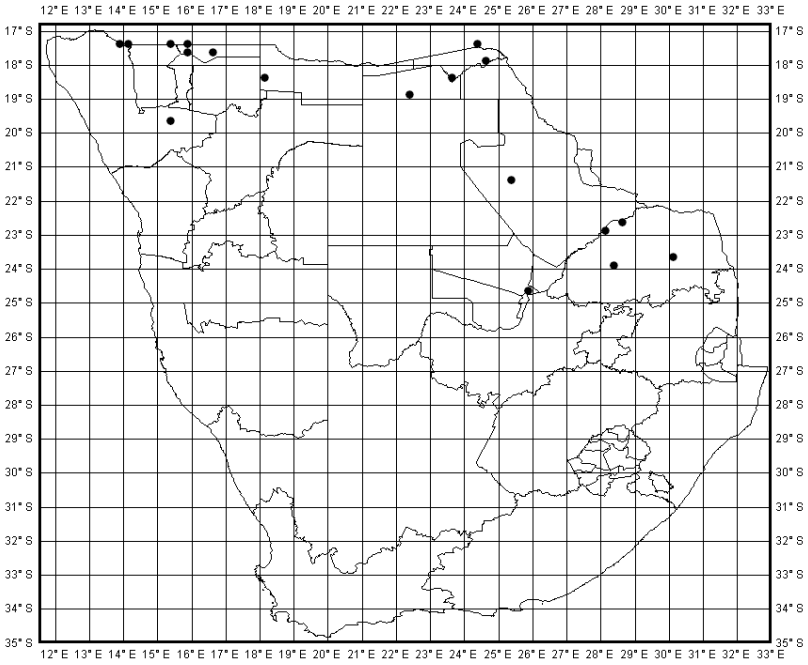


(b)

Figure 1.5: *Ficus glumosa* (a) (Photographer: GF Sibandze) and distribution map (b) (The South African National Biodiversity Institute).

greyish-green and covered with grey warts (Venter and Venter, 2002). The plant is distributed from Angola in the north to KwaZulu-Natal in the south (Figure 1.8b) and grows in woodlands (Venter and Venter, 2002). The fruit infusion is drunk to induce vomiting in the treatment of diabetes mellitus and stomach troubles; while the burnt roots are used for pneumonia. A decoction of the root is used for epilepsy, headaches and earache (Amusan *et al*., 2002; Venter and Venter, 2002; Verschaerve *et al*., 2004; PP Ndlovu, personal communication). In Thailand and Ghana, *Gardenia* species are used for the treatment of malaria (Suksamrarn *et al*., 2003; Asase *et al*., 2005).

(a)



(b)

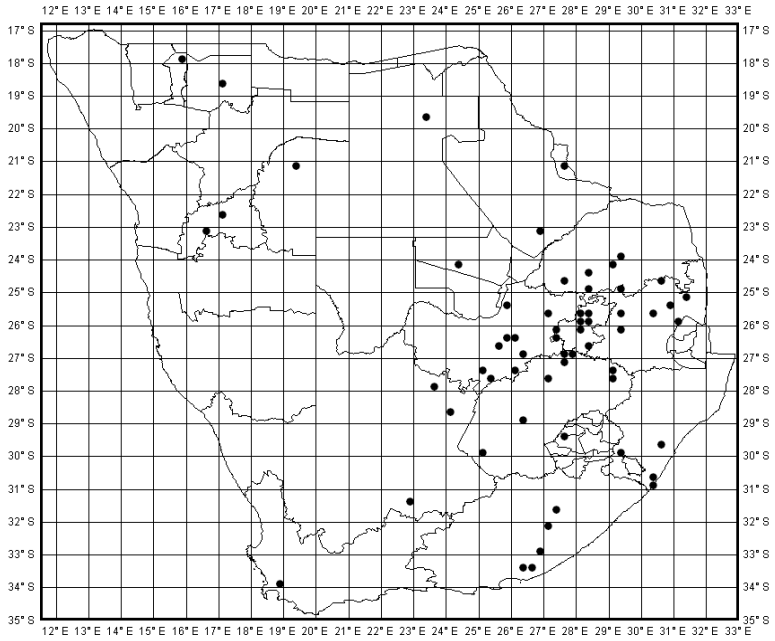
Figure 1.6: *Gardenia spatulifolia* (a) (Photographer: GF Sibandze) and distribution map (b) (The South African National Biodiversity Institute).

1.6.6 *Guilleminea densa* var. *densa* (Willd.) Moq. [syn. *Brayulinea densa* Humb. & Bonpl. ex Willd]

Guilleminea densa var. *densa* (Willd.) Moq., formerly known as *Brayulinea densa* Humb. & Bonpl. ex Willd is from the Amaranthaceae family and known as *sanama* in siSwati (PP Ndlovu; personal communication). It is a prostrate or sometimes decumbent, mat-forming perennial herb with a rootstock considerably thickened for up to about 5cm below the ground and then abruptly more slender, mat from about 7–70 cm across (Figure 1.7a). The stems are numerous from the base, much-branched, branches are opposite (or alternate by reduction of one of the pair), more or less densely white-lanate. Leaves are variable in size and shape. The inflorescence is dense, ovoid, of up to about 10 flowers and whitish in colour (Aluka, 2008). The plant is densely distributed in the north-easterly part of southern

Africa, but can also be found in parts of north Africa (Figure 1.7b). The root (tuber) is mixed with water and used to induce vomiting for the treatment of nausea (PP Ndlovu; personal communication); whilst the whole plant decoction is drunk by the Vhenda speaking people of Limpompo province in South Africa for the treatment of diarrhoea (Mathabe *et al*., 2006).

(a)



(b)

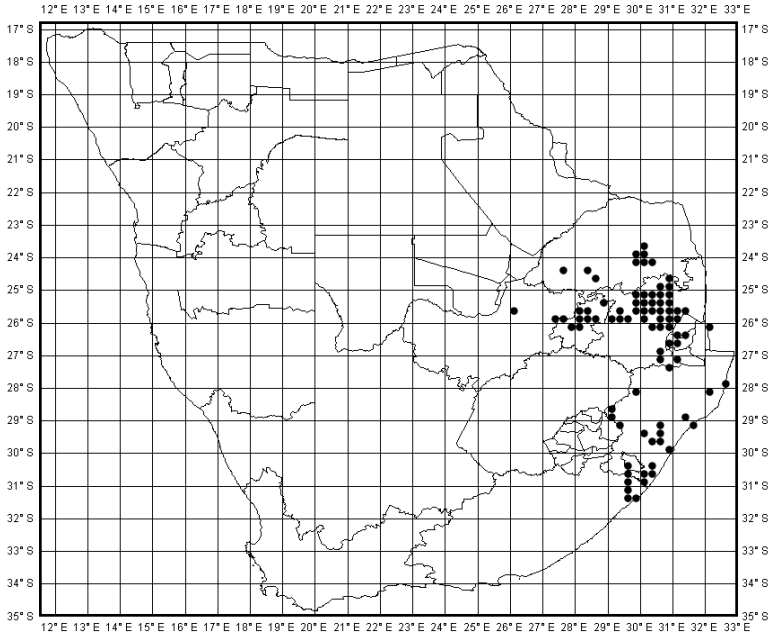
Figure 1.7: *Guilleminea densa* var. *densa* (a) (Photographer: GF Sibandze) and distribution map (b) (The South African National Biodiversity Institute).

1.6.7 *Helichrysum acutatum* DC

Helichrysum acutatum (Asteraceae) or *imboziso* in siSwati (Adeniji *et al*., 2000) is a herbaceous plant with a stout rootstalk and unbranched erect shoots, up to about 60 cm in height (Figure 1.8a). It is densely grey-woolly throughout. The leaves are few, stalklesss, tapering to a broad base and to long fin-pointed tip. The inflorescence is on a long common stalk, much-branched, compact, about 6cm in diameter. The flower heads are

bright yellow. The root is tuberous and fibrous (Compton, 1976; Adeniji *et al*., 2000), and a concoction of the roots is used as an antidote for poison (PP Ndlovu; personal communication). Other uses of this genus include the treatment of topical infections, respiratory ailments and as a dressing in circumcision rites (Amusan *et al*., 2005; van Vuuren *et al*., 2006). *Helichrysym* species (leaves and whole plant) are used for cough and tuberculosis (Watt and Breyer-Brandwijk, 1962). This genus has been reported to posses anticancer and antimycobacterial activity (Fouche *et al*., 2008; McGaw *et al*., 2008). *Helichrysum acutatum* has a wide distribution along the north-easterly region of southern Africa (Figure 1.8b).

(a)



(b)

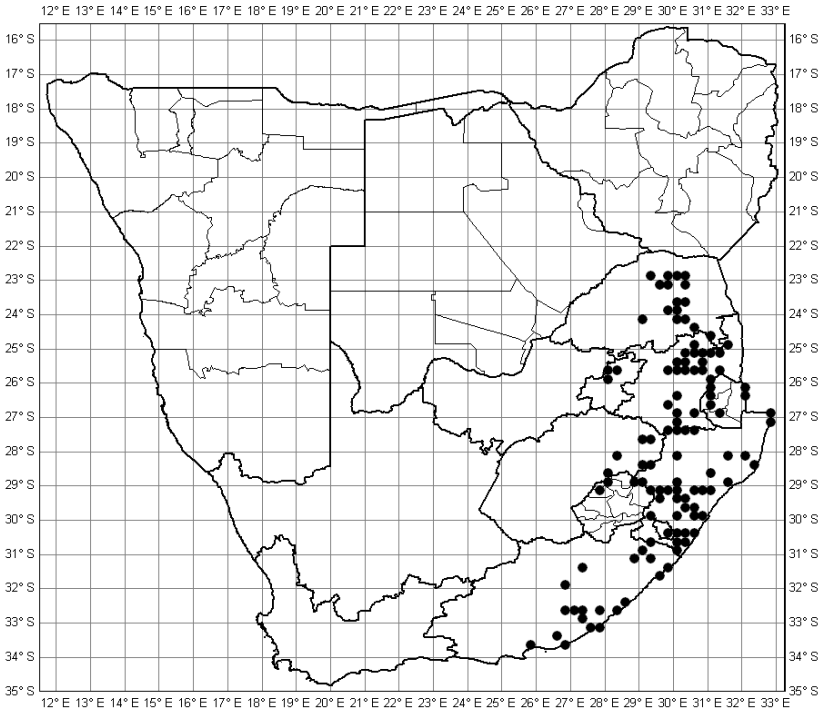
Figure 1.8: *Helichrysum acutatum* (a) (Photographer: GF Sibandze) and distribution map (b) (The South African National Biodiversity Institute).

1.6.8 *Leonotis intermedia* Lindl.

Leonotis intermedia, klipdagga or *tjwalabenyoni* (siSwati), from the Lamiaceae family, is a shrub that grows from 2-5 m in height with a thick woody base (Figure 1.9a). All parts of the plant have a strong smell and the leaves are opposite each other on the stem, long and

narrow, toothed in the upper part and distinctly hairy. The flowers are bright orange and tubular and borne in characteristic rounded groups (van Wyk *et al*., 2000). It has a wide distribution in the eastern parts of South Africa (Figure 1.9b). The fruit and roots are ground into a powder and mixed with soft porridge in the treatment of cancer. A decoction of the roots or leaves of *Leonotis spp* is mainly used for toothache, snake bites, skin disease, muscular cramps, haemorrhoids, influenza, coughs, colds, indigestion, high blood pressure, bronchitis (twigs) and also smoked as a narcotic in place of *Cannabis sativa* (Watt and Breyer-Brandwijk, 1962; van Wyk *et al*., 2000; PP Ndlovu, personal communication).

(a)



(b)

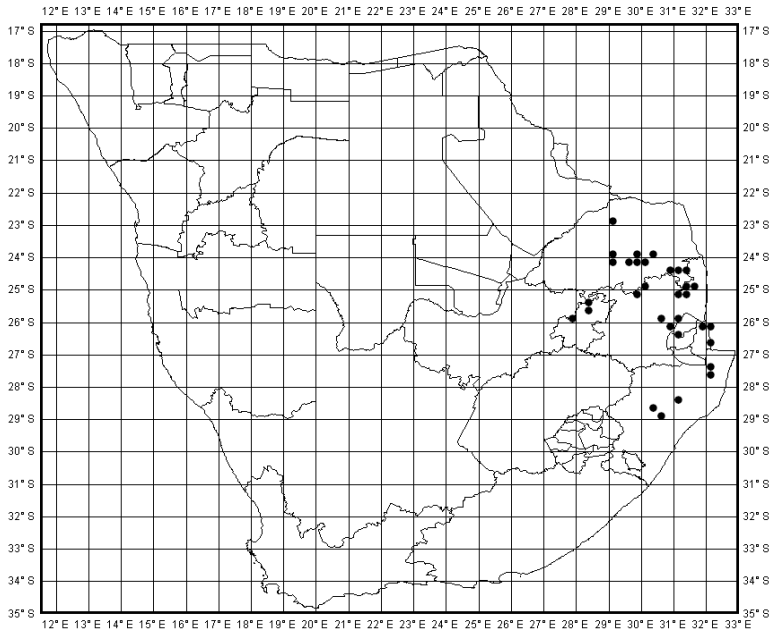
Figure 1.9: *Leonotis intermedia* (a) (Photographer: GF Sibandze) and distribution map (b) (The South African National Biodiversity Institute).

1.6.9 *Ozoroa sphaerocarpa* R.Fern. & A.Fern.

Ozoroa sphaerocarpa, currant resin tree or *imfuce* (siSwati), from family Anacardiaceae, is a deciduous tree growing to a height of 12 m with a short, bent stem and sparsely spreading

crown (Figure 1.10a). The leaves are usually in whorls of three and the fruit is ellipsoid or kidney shaped, pale green at first, becoming black and wrinkled when ripe. It flowers between September and November (Adeniji *et al*., 2000). The plant has wide distribution in deciduous woodland, bushveld and rocky hillsides (Figure 1.10b). Medicinally, the bark is used in the treatment of diarrhoea (PP Ndlovu; personal communication); while the leaf infusion is used together with *Athrixia phyllicoides* to wash wounds (Adeniji *et al*., 2000). Other species of this genus are also used medicinally, with an infusion of the leaves of *O. insignis* used to treat malaria (Asase *et al*., 2005); whereas in Zimbabwe, an infusion of the root or bark is used to treat diarrhoea, veneral diseases, tapeworm and hookworm (Rea *et al*., 2003). *Ozoroa paniculosa* is used to treat acute inflammatory infections of the chest (Watt and Breyer-Brandwijk, 1962).

(a)



(b)

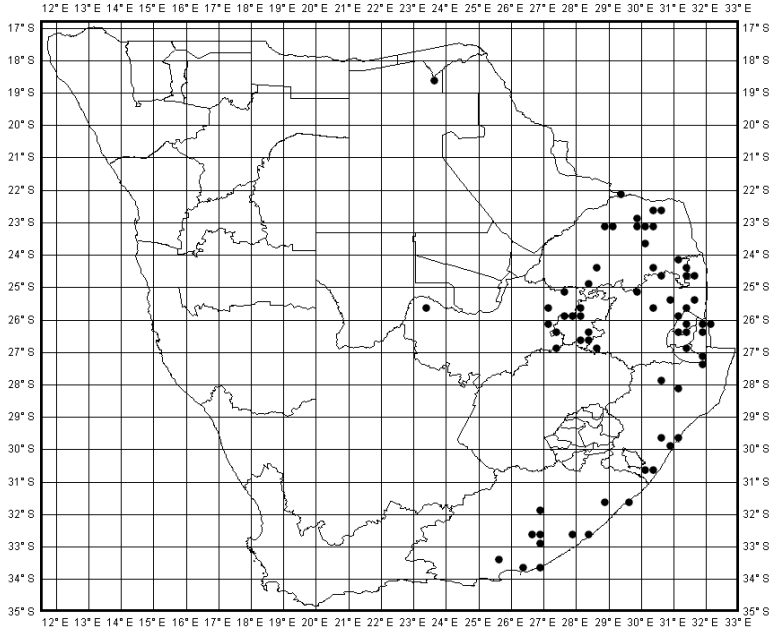
Figure 1.10: *Ozoroa sphaerocarpa* (a) (Photographer: GF Sibandze) and distribution map (b) (The South African National Biodiversity Institute).

1.6.10 *Priva meyeri* Jaub and Spach var *meyeri*

Priva meyeri or *sanama* (siSwati), from the Verbenaceae family, is a herbaceous plant, becoming woody at base and thinly rough-hairy throughout (Figure 1.11a). Its leaves are

opposite or scattered and the leaf stalk is about 1.3 cm long. The blade is triangular and broadest just above the base. Its inflorescence is terminal, elongated and indefinite. The followers are well spaced in axils of minute bracts. The corolla is mauve or white and five-lobed (Compton, 1976). The plant is distributed in the eastern part of Africa, from Tanzania in the north to Eastern Cape in the south (Figure 1.11b). The leaf infusion is traditionally used for sores and as ear drops for otitis media (Amusan *et al.*, 2005; PP Ndlovu; personal communication). Another species from this genus, *P. cardifolia* Druce is used for inflammation of the eyeball (Watt and Breyer-Brandwijk, 1962).

(a)



(b)

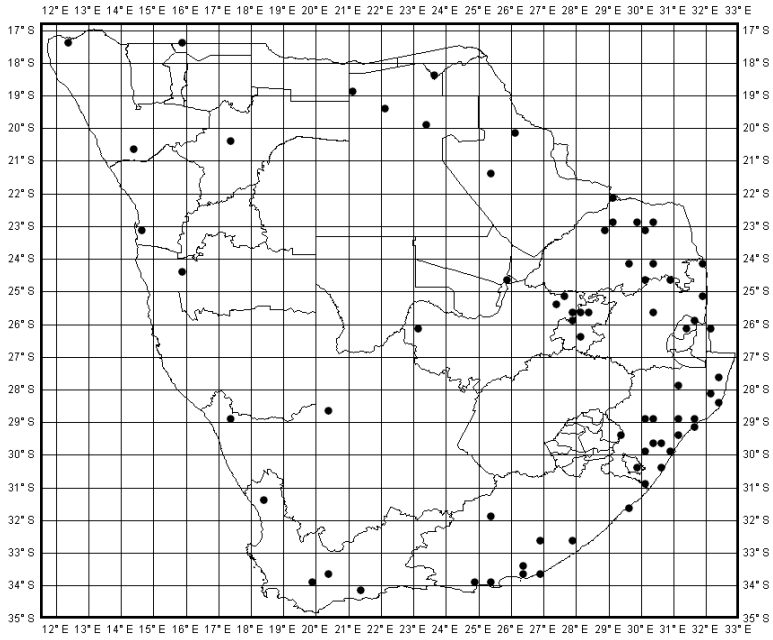
Figure 1.11: *Priva meyeri* (a) (Photographer: GF Sibandze) and distribution map (b) (The South African National Biodiversity Institute).

1.6.11 *Ricinus communis* L.

Ricinus communis, castor oil plant or *umhlafutfo* (siSwati), from the Euphorbiacea family is a large shrub of up to 4 m in height with very large, hand-shaped leaves on long, stout leaf stalks (Figure 1.12a). The flower clusters appear near the tip of the branches and the

fruits are three-lobed capsules with spine-like projections on their surfaces (van Wyk *et al*., 2000). This plant is exotic and believed to be indigenous to north-east Africa and India, but has a wide distribution in tropical areas (Figure 1.12b). It occurs predominantly as a weed on previously disturbed lands. The plant leaf infusion is medicinally administered orally or as an enema for the treatment of sores, while the unbroken seed is used for stomach-ache, toothache, sores, boils in children, headache, rheumatism and as a purgative (Watt and Breyer-Brandwijk, 1962; van Wyk *et al*., 2000; Amusan *et al*., 2005).

(a)



(b)

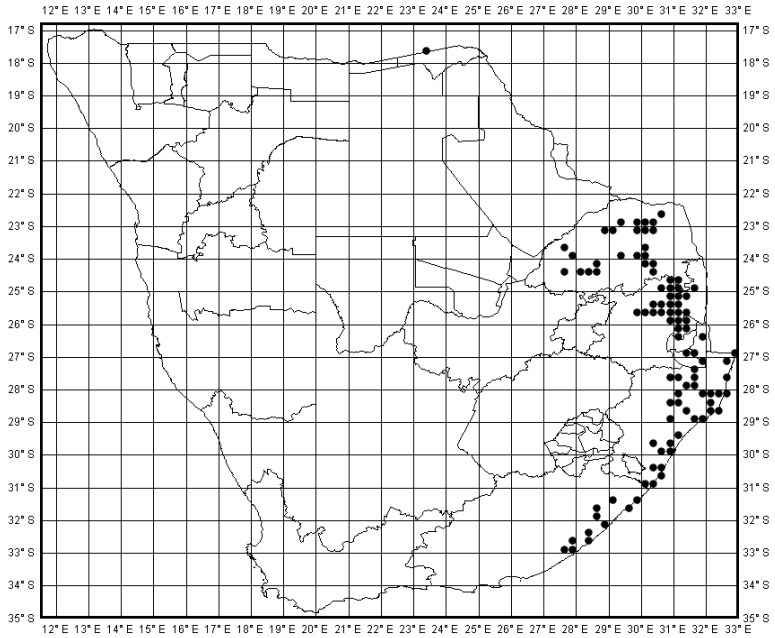
Figure 1.12: *Ricinus communis* (a) (Photographer: GF Sibandze) and distribution map (b) (The South African National Biodiversity Institute).

1.6.12 *Syzygium cordatum* Hochst ex C Krauss

Syzygium cordatum, water berry or *umncozi* (siSwati), from the family Myrtaceae, is an evergreen tree and grows up to 20 m in height (Figure 1.13a), with broad leaves near the end of the branches and are sometimes almost circular in shape with a bluish-green colour. The flowers are cream to pink in colour and appear between August and November. The

fruit is an edible berry that is egg-shaped and red to dark purple in colour. Its wood is used for boat-building (van Wyk *et al.*, 2000; Venter and Venter, 2002). The plant is distributed from Kenya to the Eastern Cape in the south (Figure 7.13b). In South Africa, it grows in the eastern and north-eastern parts and found along streams in riverine bush and forest. Medicinally the bark is used for diarrhoea, while the leaf, root and bark are used as an emetic, and also for the treatment of stomach trouble and respiratory ailments, including tuberculosis (van Wyk *et al*., 2000; Venter and Venter, 2002).

(a)



(b)

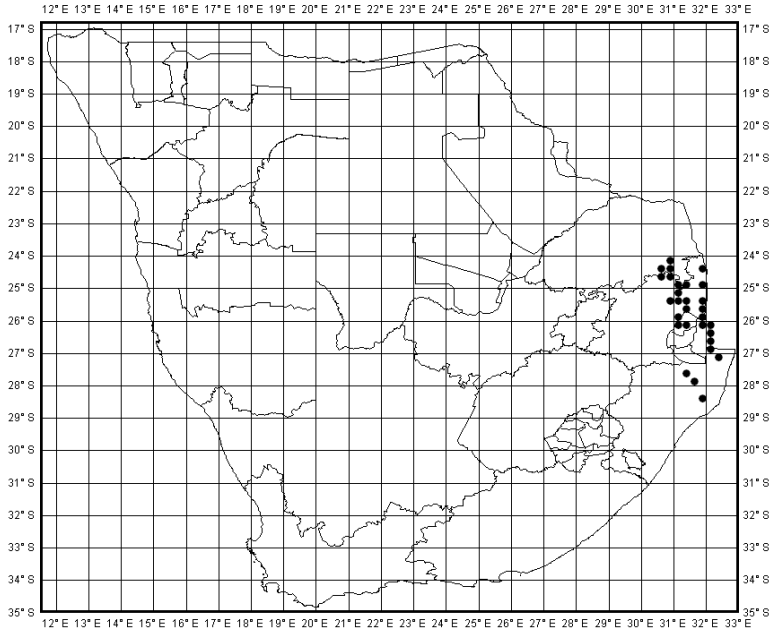
Figure 1.13: *Syzygium cordatum* (a) (Photographer: GF Sibandze) and distribution map (b) (The South African National Biodiversity Institute).

1.6.13 *Terminalia phanerophlebia* Eng. & Diels

Terminalia phanerophlebia, silver leaf tree or *emangwe lamnyama* (siSwati), from the family Combretaceae, is a small to medium-sized tree of about 5-10 m in height with an erect trunk and wide spreading crown (Figure 1.14a). The silver-grey leaves are clustered at the tops of the branchlets, erect and more or less hairy when mature. The flowers are small and yellowish, with an unpleasant smell, and the fruit is pendulous (Neuwinger,

1996; van Wyk *et al*., 2000). *T. phanerophlebia* has a small distribution area, found in savannah lands and grows in sandy soils (Figure 7.14b). The bark infusion is drunk for the treatment of body pains and pneumonia. The root extract or decoction of many *Terminalia spp*. is used for diabetes, stomach problems, schistosomiasis, gonorrhoea, syphilis, diarrhoea, general body weakness, sore throat, colic and skin disease (Neuwinger, 1996; van Wyk *et al*., 2000; Amusan *et al*., 2005). The genus has been reported to possess antimycobacterial properties (McGaw *et al*., 2008).

(a)



(b)

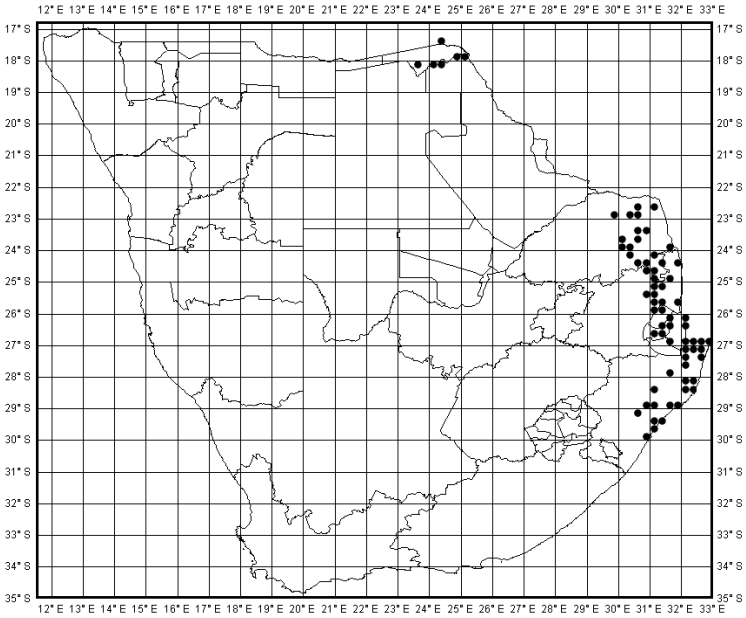
Figure 1.14: *Terminalia phanerophlebia* (a) (Photographer: GF Sibandze) and distribution map (b) (The South African National Biodiversity Institute).

1.6.14 *Trichila emetica* Vahl subsp. *emetica*

Trichilia emetica, Natal mahogany or *umkhuhlu* (siSwati), from the family Meliaceae, is a large densely leafed evergreen tree, which grows to a height of about 20 m (Figure 1.15a). The leaves are hairless above with thin hairs below. The fruit is shortly stalked, round and downy (van Wyk *et al*., 2000; Venter and Venter, 2002). This plant has a wide distribution

from Sudan to KwaZulu-Natal in the south (Figure 7.15b) and grows in coastal, riverine and gallery forests. The bark is soaked in warm water and used to treat joint pains, nausea and vomiting, malaria, dysentery, fever, haematuria, urethral discharge and abdominal pain; whilst the bark or leaf is administered as an enema to treat a sore back. The seed oil is used to treat rectal ulceration and rheumatism, and the leaf or fruit poultice is used for bruises and eczema. This plant possesses antimicrobial and anti-inflammatory properties (Watt and Breyer-Brandwijk, 1962; Adeniji *et al*., 2000; van Wyk *et al*., 2000; Venter and Venter, 2002; Amusan *et al*., 2005; Greyid *et al*., 2005).

(a)



(b)

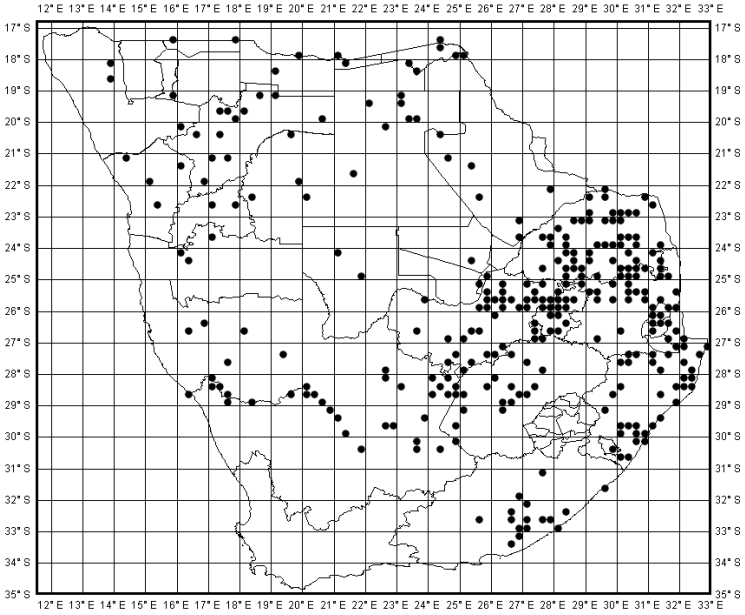
Figure 1.15: *Trichilia emetica* (a) (Photographer: GF Sibandze) and distribution map (b) (The South African National Biodiversity Institute).

1.6.15 *Ziziphus mucronata* Willd subsp. *mucronata*

Ziziphus mucronata, buffalo thorn or *umlahlabantfu* (siSwati), from the family Rhamnaceae, is a deciduous tree that can grow up to a height of 17 m and has an open round to spreading crown. The leaves are alternate, simple, smooth and shiny (Figure 1.16a). The flowers are clusters in the leaf axils, yellowish green in colour with short

flower stalks, and appear between October and April. The fruit is a round, reddish brown glossy drupe (Venter and Venter, 2002). It has a wide distribution in Africa, from Arabia to Western Cape in the south (Figure 7.16b) and grows mostly in woodland and wooded grassland. The bark powder is used for body pains and cough; while the leaf poultice is used for the treatment of chest troubles, boils and other septic swellings. The root infusion is used for treating dysentery, gonorrhoea and sores (Watt and Breyer-Brandwijk, 1962; Neuwinger, 1996; Venter and Venter, 2002; Amusan *et al*., 2005). Other *Ziziphus* species are reported to possess antidiarrhoeal activity (Tetali *et al*., 2009). The species has been reported to possess antihelmintic and anti-inflammatory activity (McGaw and Eloff, 2008).

(a)



(b)

Figure 1.16: *Ziziphus mucronata* (a) (Photographer: GF Sibandze) and distribution map (b) (The South African National Biodiversity Institute).

1.7 Rationale and motivation of study

With the increasing prevalence of antibiotic resistant microorganisms, there arises a need for research into more efficacious alternatives. Traditional medicine is one of these alternatives, as there have been numerous reports on the successful use of plants by

indigenous peoples to treat various infections such as colds, influenza, diarrhoea, dysentery, schistosomiasis, elephantiasis and malaria. (Watt and Breyer-Brandwijk, 1962; van Wyk *et al*., 1997; Amusan *et al*., 2002; Agunu *et al*., 2005; Mathabe *et al*., 2006; Mukherjee *et al*., 1998; Alanis *et al*., 2005; Gutiérrez *et al*., 2007; Samie *et al*., 2005). A further motivating factor is the success of ethnopharmacological research in the past century; which has led to the development of plant-derived drugs such as artemisinin, aspirin, atropine, colchine, digoxin, ephedrine, morphine, physostigmine, pilocarpine, quinine, vincristine and vinblastine (Gilani and Rahman, 2005). It is with these considerations that the present study was designed with the aim to search for new and alternative sources of drugs for the treatment of infectious diseases such as malaria, as well as to provide scientific proof for the efficacy claimed by traditional healers in the treatment of various diseases.

1.8 Objectives of the study

- Fifteen medicinal plants used traditionally in Swaziland (Section 1.4) were selected and screened for the following pharmacological activities:
  - antimalarial activity using the tritiated hypoxanthine incorporation assay,
  - antimicrobial properties, using the minimum inhibitory concentration (MIC) and bioautographic assays,
  - cytotoxicity using the tetrazolium-based 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method and red blood cell haemolysis,
  - anti-oxidant properties, using the 2,2-diphenyl-1-picryl-hydrazil (DPPH) free radical-scavenging activity, lipid peroxidation and metal chelating activity. This was correlated to the total phenolic and flavonoid content of the extracts.
- The more active crude extracts were characterised by thin layer chromatography (TLC) and ultra-performance liquid chromatography (UPLC).
- To determine the type of pharmacological interactions existing:
  - Between three plants which are traditionally used in combination for the treatment of diarrhoea, using the MIC assay and tested against *Escherichia coli*.
  - Between plant extracts which are most active against *Plasmodium falciparum* and quinine due to the possibility of drug interactions when using them concurrently. This was tested using the tritiated hypoxanthine incorporation assay and the MTT method for cytotoxicity.
- To provide a scientific basis for the traditional use of indigenous knowledge of these plants and compile plant monographs for the fifteen plants studied.

CHAPTER 2: PLANT COLLECTION AND EXTRACTION

2.1 Species selection

Fifteen plants were selected on the basis of their ethnomedical history, as listed in Table 2.1. A concise description and photographs of each species are shown in the monographs in Appendix B. Previous ethnobotanical investigations of the Kingdom of Swaziland done by the Swaziland Institute for Research in Traditional Medicine, Medicinal and Indigenous Food Plants (SIRMIP) were a guide to plant selection (Amusan *et al*., 2002). A literature review on the plants proposed to be included in the study yielded little or no previous research on them.

2.2 Collection of plant material

Traditional healers were consulted to identify the plants they use before collection from the wild. Thereafter, a botanist Mr GM Dlamini, positively identified them. The plants were collected from the Manzini region in Swaziland between March and April 2006 (Table 2.1). Botanical identification of the plants was done by the Malkerns Research Station, Ministry of Agriculture and Cooperatives in Swaziland and voucher specimens (voucher numbers listed in Table 2.1) deposited with the Department of Pharmacy and Pharmacology, University of the Witwatersrand, Johannesburg. Details of locality were recorded using a Global Positioning System (GPS; Table 2.1). From each plant collected either the roots, leaves, stem, flowers, tuber or bulb were harvested depending on the part that is traditionally used. The plants were collected in accordance with the manner that the traditional healer would collect them for a patient, for example, collection of the lower part of the bark and not removing all the bark around the plant. This, we were told, was because the lower end of the bark was more potent than the upper or newer bark Also, collecting on one side rather than around the stem would ensure that the tree did not die as a result of the collection.

2.3 Extraction process

In traditional medicine, the norm is to use an aqueous solvent to prepare the medicine, however, it was decided for the purpose of this study to use an organic solvent to extract the secondary metabolites as previous studies have shown the organic solvent to be more potent as

Table 2.1: Medicinal plants selected for investigation in this study.

Family Name	Scientific Name	Plant type	Plant parts collected	Location			Collection Date	Voucher number
				Area	GPS reading	Altitude		
Astereaceae	*Berkheya setifera* DC.	Herb	Flower, stem/root, leaves	Hhelehhele	26.49023°S, 031.44882°E	697 m	March 2006	GM007
Rubiaceae	*Breonadia salicina* (Vahl) Hepper & J.R.I. Wood	Tree	Leaves, bark	Bhunya	26.54145°S, 031.00538°E	925 m	March 2006	GM012
Mimosaceae	*Dichrostachys cinerea* (L.) Wight & Arn.	Tree	Fruit, leaves	KaShali (Manzini)	26.54669°S, 031.36883°E	467 m	March 2006	GM014
Moraceae	*Ficus glumosa* Delile	Tree	Fruit, leaves, bark	Moti	26.70728°S, 031.41334°E	329 m	April 2006	GM004
Rubiaceae	*Gardenia volkensii* K. Schum subsp. *spatulifolia*	Tree	Fruit, leaves	Moti	26.71151°S, 031.41209°E	342 m	April 2006	GM001
Amaranthaceae	*Guilleminea densa* var. *densa* (Willd.) Moq.	Herb	Whole plant	Moti	26.70428°S, 031.42282°E	334 m	April 2006	GM009
Astereaceae	*Helichrysum acutatum* DC.	Herb	Leaves, roots	Bhunya	26.56151°S, 031.00286°E	1197 m	March 2006	GM010
+Lamiaceae	*Leonotis intermedia* Lindl.	Herb	Flower, stem	Bhunya	26.55508°S, 031.00080°E	1087 m	March 2006	GM008
Anacardiaceae	*Ozoroa sphaerocarpa* R.Fern. & A.Fern.	Tree	Fruit, leaves, bark	Moti	26.71127°S, 031.41359°E	339 m	April 2006	GM002
Verbenaceae	*Priva meyeri* Jaub and Spach var. *meyeri*	Herb	Whole plant	Bhunya	26.55508°S, 031.00080°E	1087 m	March 2006	GM015
Euphorbiaceae	*Ricinus communis* L.	Herb / Shrub	Fruit, leaves, stem	Bhunya	26.54145°S, 031.00538°E	925 m	March 2006	GM011
Myrtaceae	*Syzygium cordatum* Hochst ex C Krauss	Tree	Leaves, bark	Malkerns	26.54871°S, 031.18032°E	754 m	March 2006	GM013
Combretaceae	*Terminalia phanerophlebia* Eng. & Diels	Tree	Leaves, bark	Moti	26.71127°S, 031.41359°E	339 m	April 2006	GM003
Meliaceae	*Trichila emetica* Vahl subsp. *Emetic*	Tree	Leaves, bark	Moti	26.70436°S, 031.41931°E	319 m	April 2006	GM005
Rhamnaceae	*Ziziphus mucronata* Willd subsp. *Mucronata*	Tree	Leaves, fruit, bark	Zakhele	26.50191°S, 031.36638°E	614 m	March 2006	GM006

most of the components of the plants are easily extracted in the phase compared to the aqueous extractant (ref)

The plant parts were air-dried separately in the shade, crushed into powder using a commercial blender and a known quantity extracted with dichloromethane (Merck): methanol (Sigma) (1:1) solvent for 48 hours at room temperature. The solvent was changed twice during this period. The extract was then taken to dryness using a Büchi Rotavapor (Büchi Rotavapor R-114) (Figure 2.1). Thereafter, it was air-dried in a fume hood and stored in airtight containers at -20^{o}C until used. The percentage yield for each part is listed in Table 2.2 and ranges from 0.5 to 12.2%.

There was variability in the percent yield of the plant parts with the stem generally giving the least extract yield. This may be because the stems of most plants act as translocation organs and not as storage organs and as expected will have fewer compounds in them. The least yield (0.5%) was obtained from the fruit of *Z. mucronata*, followed by the stem of *L. intermedia* (1.2%), stem/root of *B. setifera* and stem of *R. communis* (1.6%). The highest yield was from the bark of *B. salicina* (12.2%) followed by the leaves of *G. spatulifolia* (10.8%), fruit of *B. densa* (10%) and bark of *S. cordatum* (9.1%).

Figure 2.1: Büchi Rotavapor used to take extracts to dryness (Photographer: GF Sibandze).

Table 2.2: Percentage yield for plant parts after extraction.

Scientific name	Plant part	Percentage (%) yield (w/w)
G. densa	whole plant	3.3
O. sphaerocarpa	fruit	10.0
	leaves	6.6
	bark	5.0
B. setifera	flower	7.6
	stem/root	1.6
	leaves	4.3
H. acutatum	leaves	3.7
	root	4.3
T. phanerophlebia	leaves	3.6
	bark	4.1
R. communis	fruit	4.2
	leaves	2.9
	stem	1.6
L. intermedia	flower	4.6
	stem	1.2
T. emetica	leaves	7.2
	bark	6.6
D. cinerea	fruit	7.5
	leaves	8.6
F. glumosa	fruit	3.4
	leaves	4.2
	bark	7.8
S. cordatum	leaves	6.9
	bark	9.1
Z. mucronata	leaves	8.6
	fruit	0.5
	bark	8.1
B. salicina	leaves	8.4
	bark	12.2
G. spatulifolia	fruit	4.8
	leaves	10.8
P. meyeri	whole plant	3.9

CHAPTER 3: UPLC-MS AND HPTLC CHEMICAL FINGER-PRINTING OF EXTRACTS

3.1 Introduction

Plants extracts are complex mixtures of numerous primary and secondary metabolites. The strength of the plant's biological activities is dependant on the diversity and quantity of its secondary metabolites such as tannins, terpenoids, alkaloids, polyphenols, steroids and saponins. In the use of plant extracts as medicine, the preparation is a complex mixture of active constituents which should be present in a constant concentration. This standardization can be achieved by quantitative determination of the marker substances (Geyid *et al*., 2005; Hamburger and Hostettmann, 1989). Chromatography, which is a means of separating two or more substances by distribution between a stationary and mobile phase (Finar, 1975), can be used to generate a fingerprint of the plant extract and provide a base for standardization procedures. It can also be used to identify the chemical constituents of the extract for possible isolation and testing of active constituents.

3.1.1 Thin layer chromatography and high-performance thin layer chromatography

Thin layer chromatography (TLC) is a quick, simple inexpensive procedure which uses a chromoplate as a column i.e. the TLC plate. The chromoplate is a sheet of glass, metal or plastic, coated with a thin layer of solid adsorbent, usually silica gel (SiO_2 x H_2O) or alumina (Al_2O_3 x H_2O). The plate is spotted with a small amount of the analyte near the bottom and placed vertically in a suitable solvent in a closed tank. Development of the chromatogram occurs by capillary movement of the solvent up the adsorbent layer. In principle, the components of the analyte will differ in their solubility and in the strength of their adsorption to the adsorbent, resulting in some components being carried further than others (Finar, 1975; Heinrich *et al*., 2004; CUBoulder Organic Chemistry, 2008). The more polar compounds tend to remain at the bottom of the TLC plate, whilst the less polar compounds migrate faster upwards on the plate.

High-performance thin layer chromatography (HPTLC) uses the same principle as the conventional TLC method, differing only in the fact that the HPTLC plate uses an adsorbent with a homogenous particle size (approximately 5μm) with a narrow particle size distribution.

HPTLC is thus superior to the conventional TLC in terms of obtaining better and clearer separation of the compounds (Qian *et al*., 2007; Kaur *et al*., 2008)

3.1.2 High-performance liquid chromatography and ultra-performance liquid chromatography

High-performance liquid chromatography (HPLC) is a form of liquid chromatography used to separate compounds that are dissolved in solution. HPLC instruments consist of a reservoir of mobile phase, a pump, an injector, a separation column and a detector. Compounds are separated by injecting a spot or band of the sample mixture onto the column. Separation is achieved because the components of the mixture pass through the column at different rates due to differences in their partitioning behaviour between the mobile liquid phase and the stationary phase (Science Hypermedia, 1996). This is a rapid and reliable technique for analysis of complex mixtures (Hamburger and Hostettmann, 1989), and is also a powerful technique for fingerprinting biologically active extracts. HPLC instruments are normally coupled to a computer which has a library that allows for easier identification of compounds that have been previously isolated (Heinrich *et al*., 2004).

Ultra-performance liquid chromatography (UPLC) on the other hand is a more advanced form of HPLC which employs smaller particles (packed in the column), speed and peak capacity. These characteristics have resulted in an instrument with high resolution and that is more efficient, thus the UPLC detection is two to three times higher than that of the HPLC. A UPLC system can either be coupled to a mass spectrometry, nuclear magnetic resonance, refractive index, near-infra red or UV-visible detector (Swartz, 2005; Li *et al*., 2006; De Villiers *et al*., 2006).

The aim of this chapter was to fingerprint the most active extracts by using both HPTLC and UPLC and provide a base for further study to possibly isolate the active constituents in the extract.

3.2 Materials and methods

Based on the preliminary antimicrobial, anti-oxidant and antimalarial activities, 22 of the 33 extracts tested in this study were selected for TLC analysis. These were the leaves and bark of

T. phanerophlebia, *S. cordatum*, *B. salicina*, *Z. mucronata* and *T. emetica*, the fruit and leaves of *D. cinerea* and *R. communis*, the bark, fruit and leaves of *F. glumosa* and *O. sphaerocarpa*, the leaves of *G. spatulifolia* and the whole plant of *P. meyeri*. Sixteen extracts were selected for UPLC analysis and included the fruit and leaves of *D. cinerea* and *G. spatulifolia*, the leaves and bark of *S. cordatum* and *B. salicina*, the fruit, leaves and bark of *O. sphaerocarpa*, the fruit, leaves and stem of *R. communis* and the root and leaves of *H. acutatum*.

3.2.1 High-performance thin layer chromatography method

The extract (10 mg/ml) was prepared in HPLC grade methanol (E-lab Direct Limited). The TLC mobile phase was prepared using methanol: water: ethyl acetate (16.5:13.5:100). The HPTLC plates (HPTLC silica gel 60 F_{254}, 20 x 10 mm (Merck)) were run on a CAMAG high-performance thin layer chromatography (HPTLC) system at the Department of Pharmacy at the Tshwane University of Technology (Pretoria). The extract (15 µl) was spotted with a CAMAG automatic TLC sampler 4, band length 12 or 8 mm, depending on the number of samples to be placed on the plate. Thereafter, the plates were developed in a CAMAG automatic development chamber 2. Methanol was used as a rinsing solvent. The TLC mobile phase (20 ml) was used to develop the plates in a saturated chamber and the solvent front was allowed to migrate to a distance of 80 mm. The plates were then visualized using a CAMAG reprostar 3 at 254 nm, 366 nm and using white light. The reference plates were derivatized by dipping them into vanillin-sulphuric acid (200 ml; prepared in a 1:1 ratio using 1% ethanolic vanillin (Sigma-Aldrich) and 10% ethanolic sulphuric acid (Merck) (Wagner and Bladt, 1986)) using a CAMAG chromatogram immersion device III. The plates were then dried in a CAMAG TLC plate heater III at 104 ^{o}C for 5 minutes to achieve full colour development. The winCATS program was used to operate the instrument, as well as capture images of the HPTLC plates.

3.2.2 Ultra-performance liquid chromatography coupled to mass spectrometer method

3.2.2.1 Sample preparation

The dried extracts were re-suspended in 1ml HPLC grade methanol and diluted 10 times in 50% acetonitrile and 0.1% formic acid. Thereafter, the suspension was centrifuged for 5 minutes at 12000 rpm.

3.2.2.2 Assay

UPLC-MS analysis was done on a Waters Acquity Ultra Performance Liquid Chromatograph™ connected to a Waters Q-TOF Ultima™ mass spectrometer according the method described by Rautenbach *et al*. (2007) and Storbeck *et al*. (2008). Separation was achieved on a Waters UPLC BEH C18 column (2.1×50 mm, 1.7 μm spherical particles, Millipore-Waters, La Jolla, USA), using a formic acid (A) to acetonitrile (B) gradient (100% A from 0 to 10 minutes, 4 to 25% B from 10 to 15 minutes, 25 to 90% B from 15 to 30 minutes and 100% A from 30.1 to 40 minutes at a flow rate 300 μl/minutes), followed by re-equilibration of the column to initial conditions. The sample (4 μl) was introduced into the mass spectrometer after passing through the Waters Acquity UPLC™. The carrier solvent was 50% acetonitrile in 0.1% formic acid, which was delivered at a flow rate of 300 μl/minutes for each analysis. A capillary voltage of 3.5 kV with a source temperature of 100 ^{o}C and a cone voltage of 35 V was applied. The total run time per sample was 25 minutes and data acquisition was in the positive mode and results expressed as mass to charge ratio (m/z).

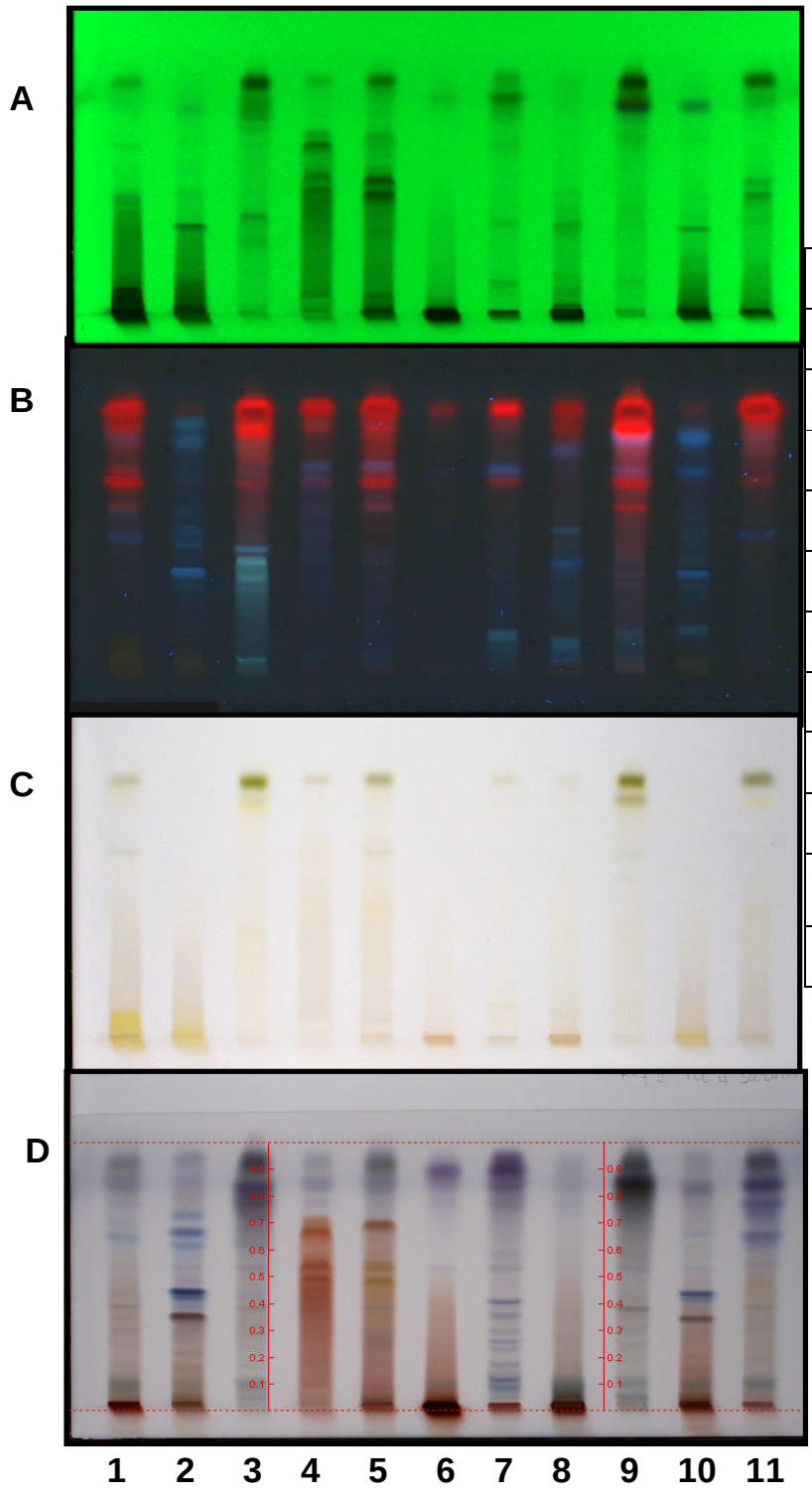
3.3 Results

3.3.1 High-performance thin layer chromatography

Figures 3.1 and 3.2 show the HPTLC chromatograms of the 22 extracts. Several compounds were visualized under UV light (254 and 366 nm), as well as after derivatization with vanillin-sulphuric acid. After derivatization, many compounds that had not been visible under UV light were observed. The dark areas visualized under the 254 nm light are unsaturated hydrocarbons containing C=C double bonds. The dark red areas at the bottom of the plates and predominantly found in the bark material are presumed to be tannins, since they are very polar and did not migrate on the plate (Figure 3.1A). The purple bands at the top of the plates are non-polar compounds, typical of aromatic compounds. These are also visible under the UV366 nm light.

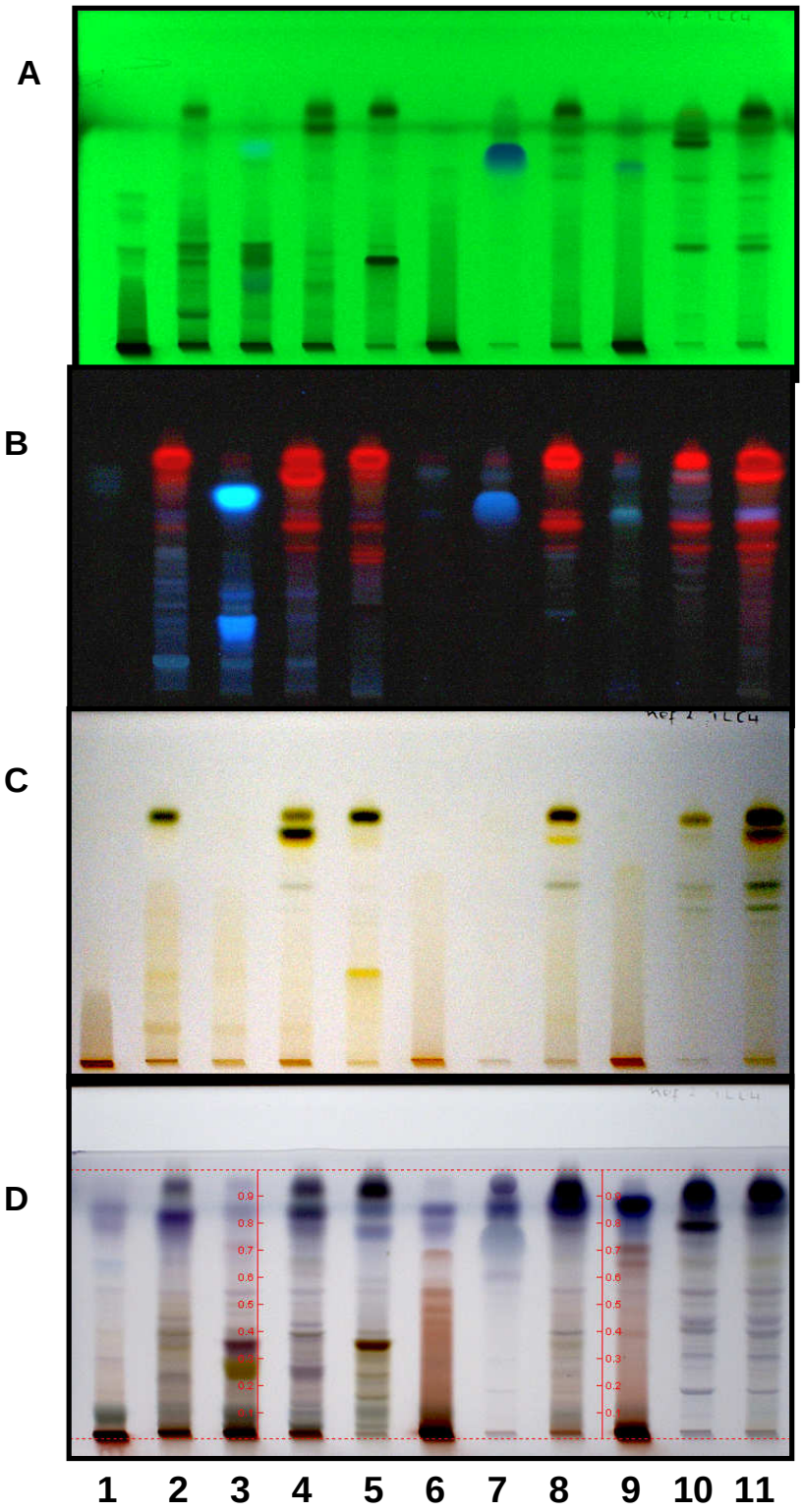
3.3.2 Ultra-performance liquid chromatography

The UPLC results are a comparison between parts of the same plant to determine if there any chemical similarities or variations in the compounds found in each extract. Figures 3.3 to 3.9



Track name	Plant sample
1	*T. phanerophlebia* leaves
2	*T. phanerophlebia* bark
3	*P. meyeri* whole plant
4	*D. cinerea* fruit
5	*D. cinerea* leaves
6	*F. glumosa* bark
7	*F. glumosa* fruit
8	*F. glumosa* leaves
9	*T. emetica* leaves
10	*T. emetica* bark
11	*S. cordatum* leaves

Figure 3.1: HPTLC plate for 11 extracts (Group 1), visualized under 254 nm (A), 366 nm (B), white light (C) and after derivatization with vanillin-sulphuric acid (D).



Track name	Plant sample
1	*S. cordatum* bark
2	*B. salicina* leaves
3	*B. salicina* bark
4	*G. spatulifolia* leaves
5	*Z. mucronata* leaves
6	*Z. mucronata* bark
7	*O. sphaerocarpa* fruit
8	*O. sphaerocarpa* leaves
9	*O. sphaerocarpa* bark
10	*R. communis* fruit
11	*R. communis* leaves

Figure 3.2: HPTLC plate for other 11 extracts (Group 2), visualized under 254 nm (A) 366 nm (B), white light (C) and after derivatization with vanillin-sulphuric acid (D.

and Table 3.1 to 3.7 are UPLC chromatograms and results showing the base peak intensity (BPI) of the different compounds in the sixteen extracts chosen for UPLC analysis. The UPLC chromatograms of all the plants were different, showing a variety of compounds in the plants. In some cases, extracts from the same plant had similar chromatograms, only differing in the relative abundance of the compound, such as the leaves and bark of *S. cordatum* (Figure 3.4); whilst others differed greatly, such as the fruit, leaves and stem of *R. communis* (Figure 3.8).

3.4 Discussion

The HPTLC chromatograms of the different plants showed the complexity of the plant extracts. Several compounds that had not been visible before derivatization (Figure 3.1C and 3.2C) or under UV light (Figure 3.1A and B and 3.2 A and B) were observed after dipping in vanillin-sulphuric acid (Figure 3.1D and 3.2D). The plants which seemed to exhibit similar compounds among their parts were *S. cordatum*, *D. cinerea* and *R. communis* (Figure 3.1D, track 4/ 5 and 11; Figure 3.2D, track 1/10 and11), otherwise the others did not show similar chromatograms. This might be due to the plants producing different classes of compounds among their own vegetative parts and also account for the varying biological activities observed in them (Chapter 4, 5 and 6). The UPLC profiles of the plants also confirm these differences, however, due to the sensitivity of this procedure, the UPLC profiles were able to show some similarities among the different plant parts.

The leaves and bark of *T. phanerophlebia* exhibited different HPTLC chromatograms, with the bark having more compounds separating than the leaves (Figure 3.1, track 1 and 2). Previous studies on the *Terminalia* genus have isolated different compounds from the plant such as terpenoidas and calchone glycosides and flavones from *T. alata* (Srivastava *et al*., 1999) and uronic acids from the gum exudates of *T. sericea* and *T. superba* (Anderson and Bell, 1974). Arjunic acid, arjungenin, arjunetin and arjunoglucoside I have been isolated from the bark of *T. arjuna* (Saxena *et al*., 2007; Manna *et al*., 2007); while Alam *et al*. (2008) isolated termiarjunoside I and termiarjunoside II from *T. arjuna*. Termilignan B and arjunic acid were isolated from *T. sericea* (Eldeen *et al*., 2008). Gum exudates of some *Terminalia* species have been reported to contain uronic acids (Douglas and Bell, 1974).

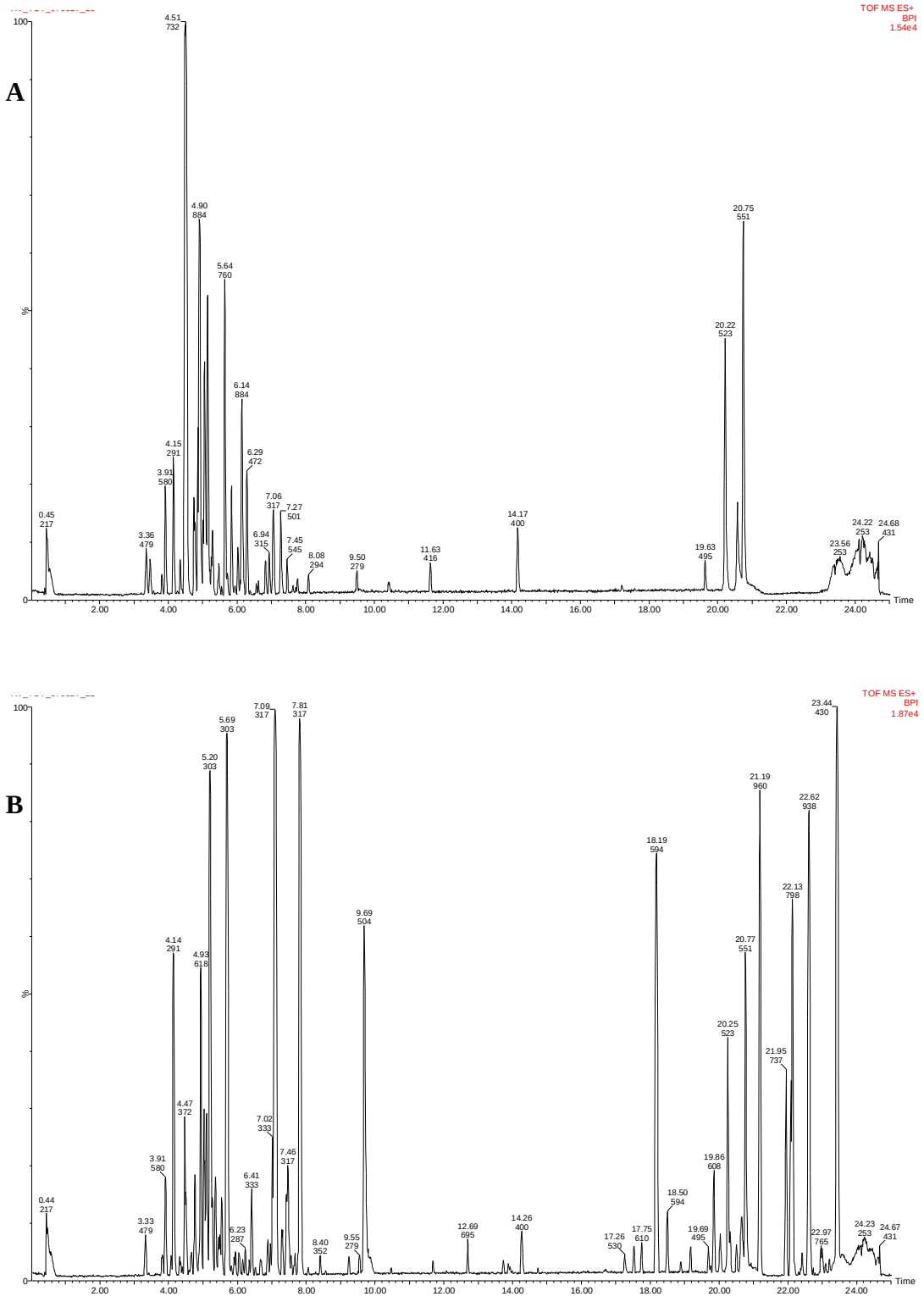


Figure 3.3: Base peak intensity chromatograms of the fruit (A) and leaves (B) of *D. cinerea*.

Table 3.1: Retention time, mass to charge ratio and relative abundance of the peak of the fruit and leaves of *D. cinerea*.

Retention time (minutes)	*m/z*	Relative intensity (%)	
		Fruit	Leaves
3.91	580	20	20
4.14/4.15	291	25	58
4.47	372		30
4.51	732	100	
4.90	884	65	
4.93	618		55
5.20	303		90
5.64	760	55	
5.69	303		95
6.14	884	35	
6.29	472	25	
7.02	333		25
7.09	317		100
7.46	317		20
7.81	317		100
9.69	504		55
18.19	594		75
19.86	608		20
20.22/20.25	523	45	45
20.75/20.77	551	65	60
21.19	960		85
21.95	737		40
22.13	798		68
22.62	938		82
23.44	430		100

Table 3.2: Retention time, mass to charge ratio and relative abundance of the peak of the leaves and bark of *S. cordatum.*

Retention time (minutes)	*m/z*	Relative intensity (%)	
		Leaves	Bark
0.42	368		22
4.16	327		35
4.36	634	25	
4.47	372	50	45
4.77	433		40
5.04	319	100	
5.04	477		48
5.25/5.28	521	35	35
5.67	303	30	
7.07/7.12	457	25	25
7.28/7.33	501	35	35
7.47/7.52	545	25	30
20.20/20.22	523	40	92
20.72/20.75	551	55	100
24.22	253		20

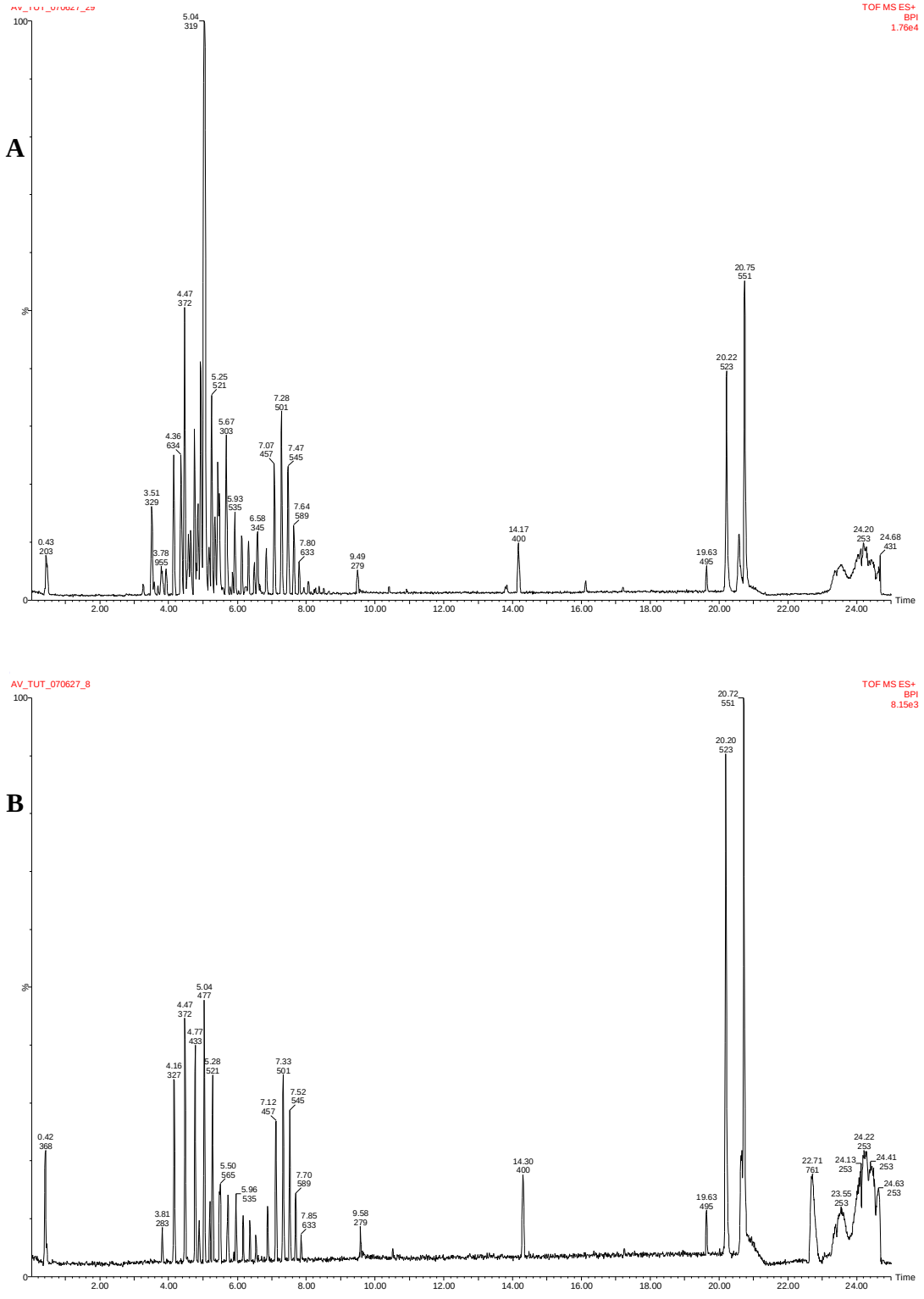


Figure 3.4: Base peak intensity chromatograms of the leaves (A) and bark (B) of *S. cordatum.*

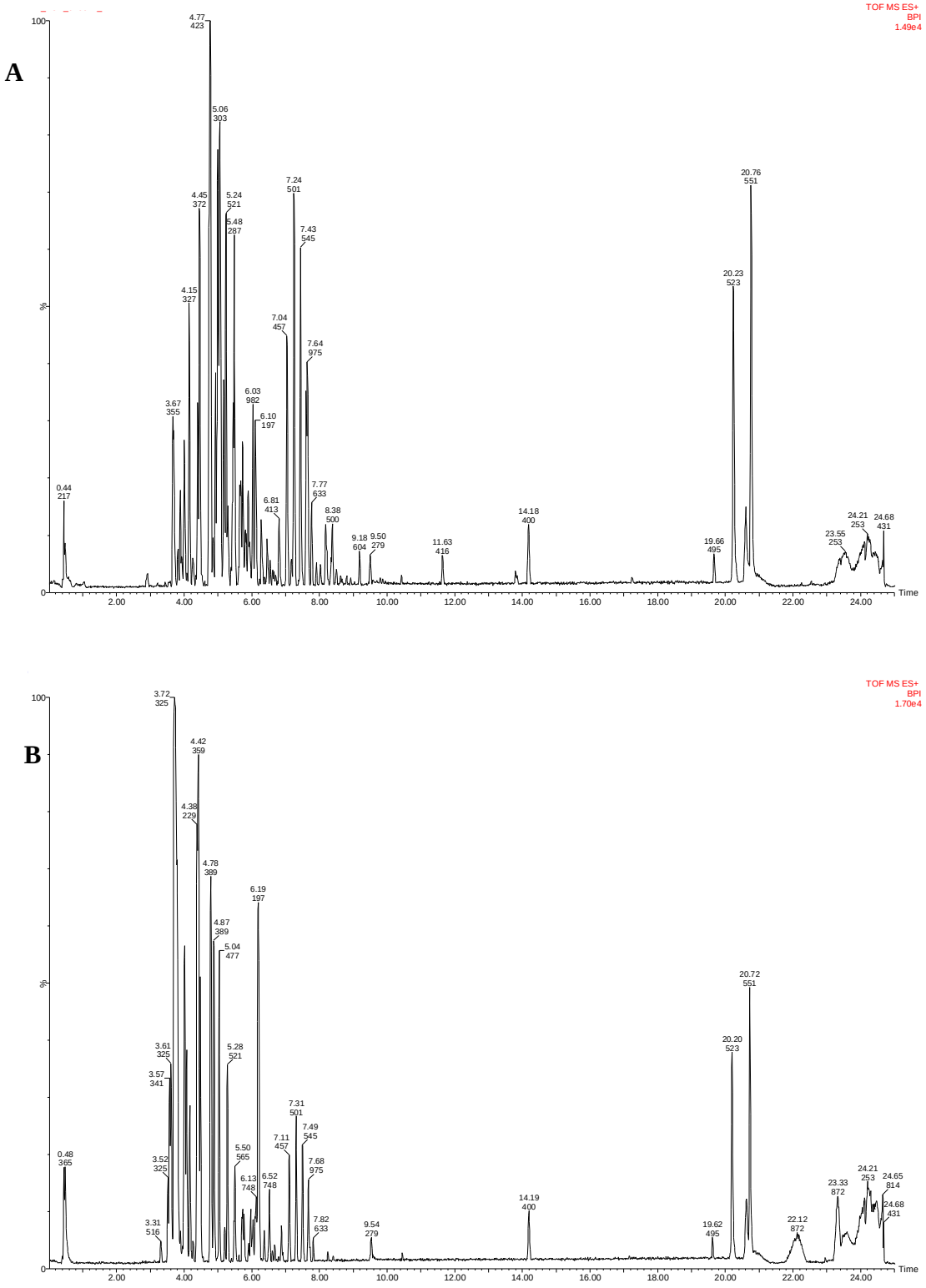


Figure 3.5: Base peak intensity chromatograms of the leaves (A) and bark (B) of *B. salicina*.

Table 3.3: Retention time, mass to charge ratio and relative abundance of the the pek of the leaves and bark of *B. salicina*.

Retention time (minutes)	*m/z*	Relative intensity (%)	
		Leaves	Bark
3.57	341		32
3.67	355	32	
3.61	325		35
3.72	325		100
4.15	327	50	
4.38	229		78
4.42	359		90
4.45	372	68	
4.77	423	100	
4.78	389		70
5.04	477		55
5.06	303	85	
5.24/5.28	521	68	35
5.48	287	65	
6.03	982	35	
6.10/6.19	197	30	65
7.04	457	45	
7.24/7.31	501	72	28
7.43	545	62	
7.64	975	40	
20.20/20.23	523	55	40
20.72/20.76	551	75	52

Table 3.4: Retention time, mass to charge ratio and relative abundance of the peak of the fruit and leaves of *G. spatulifolia*.

Retention time (minutes)	*m/z*	Relative intensity (%)	
		Fruit	Leaves
3.19	225		52
3.35	225		100
4.10	904		45
4.15/4.16	327	32	52
4.47/4.48	372	58	100
4.73	310		90
5.02/5.03	477	65	100
5.26/5.27	521	55	92
5.49/5.48	565	28	38
7.11/7.09	457	48	28
7.29/7.32	501	55	40
7.50/7.49	545	50	30
7.68	589	28	
10.62	438	32	
11.95	804	30	
17.15	516	25	
20.25/20.27	523	42	68
20.78/20.80	551	56	92
22.28	873	100	
23.36	872	100	

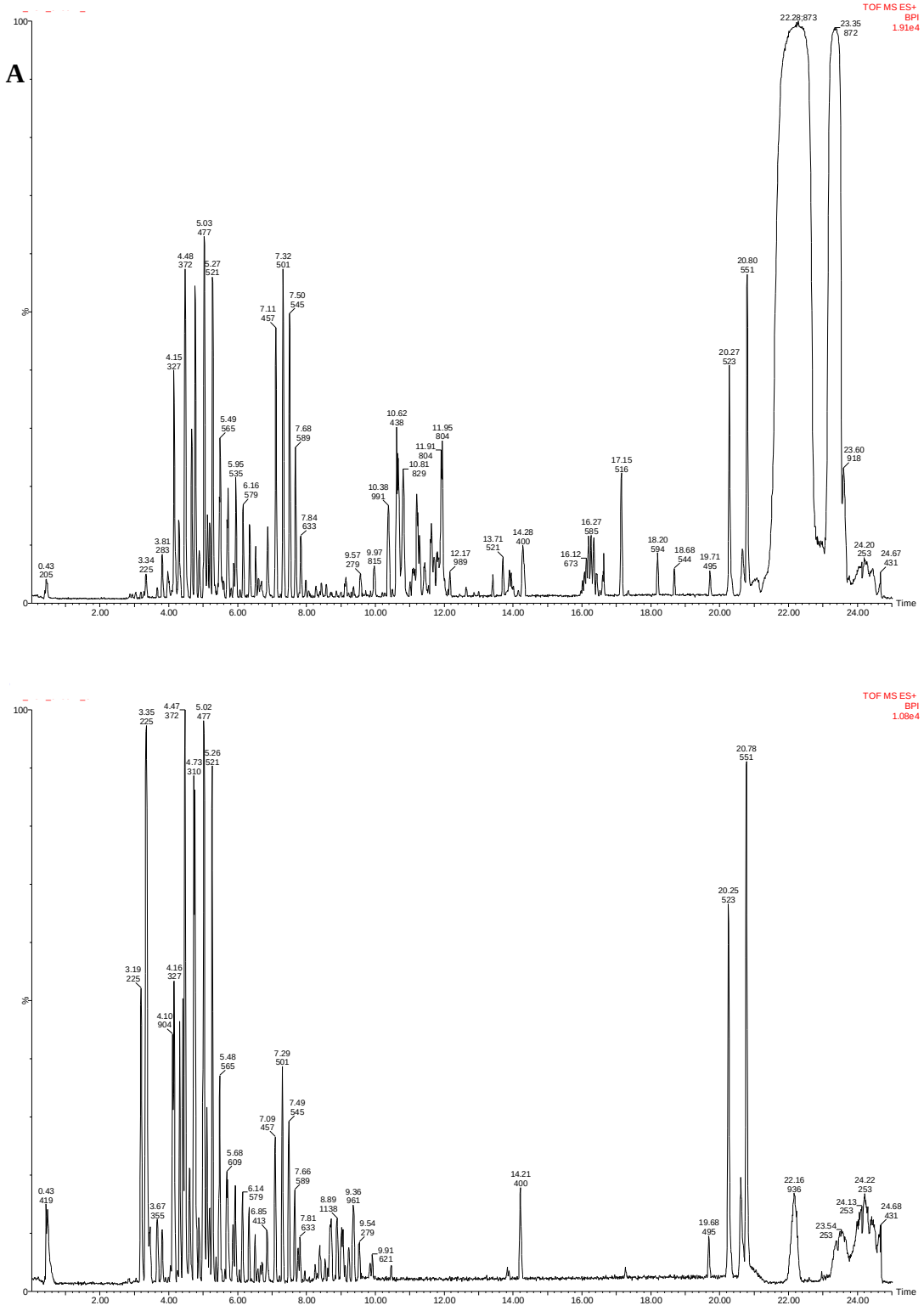
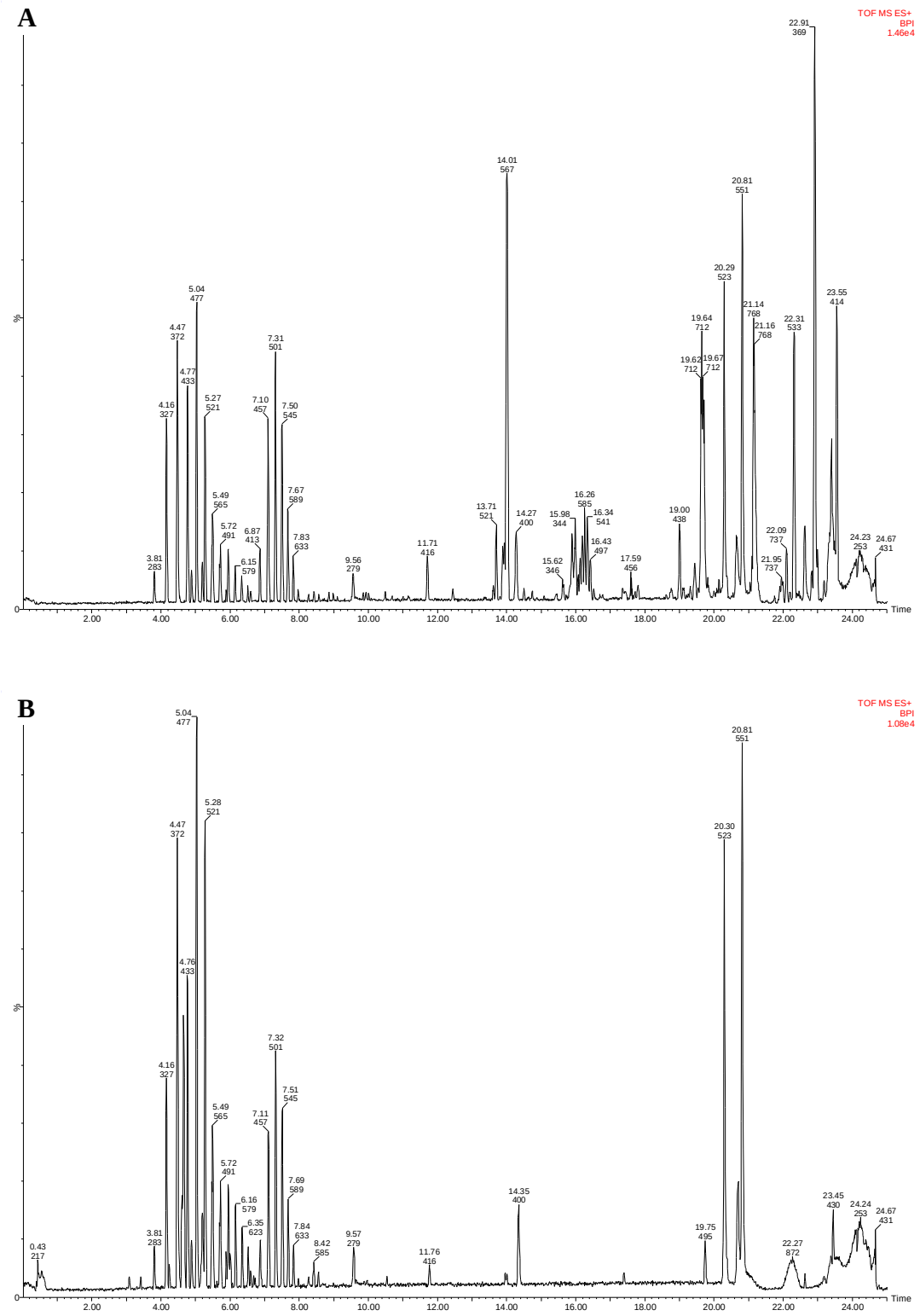


Figure 3.6: Base peak intensity chromatograms of the fruit (A) and leaves (B) of *G. spatulifolia*.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3.81 283
4.16 327
4.47 372
4.77 433
5.04 477
5.27 521
5.49 565
5.72 491
6.15 579
6.87 413
7.10 457
7.31 501
7.50 545
7.67 589
7.83 633
9.56 279
11.71 416
13.71 521
14.01 567
14.27 400
15.62 346
15.98 344
16.26 585
16.34 541
16.43 497
17.59 456
19.00 438
19.62 712
19.64 712
19.67 712
20.29 523
20.81 551
21.14 768
21.16 768
21.95 737
22.09 737
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22.91 369
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24.23 253
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5.28 521
5.49 565
5.72 491
6.16 579
6.35 623
7.11 457
7.32 501
7.51 545
7.69 589
7.84 633
8.42 585
9.57 279
11.76 416
14.35 400
19.75 495
20.30 523
20.81 551
22.27 872
23.45 430
24.24 253
24.67 431

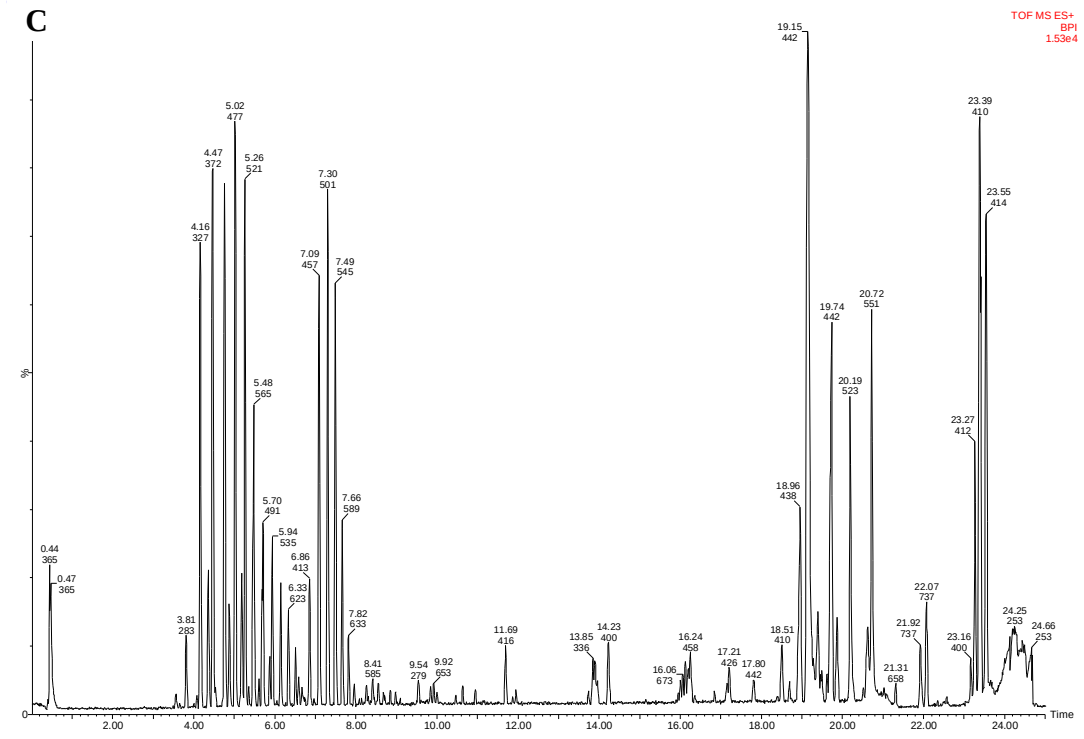
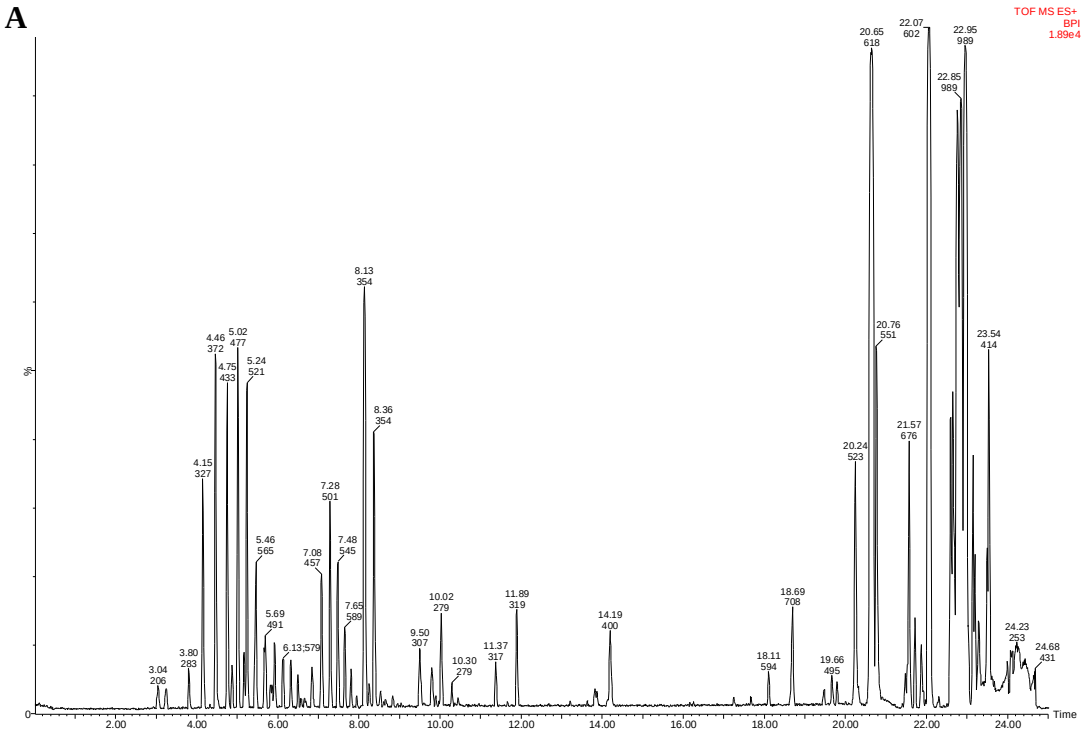


Figure 3.7: Base peak intensity chromatograms of the fruit (A), leaves (B) and bark (C) of *O. sphaerocarpa*.



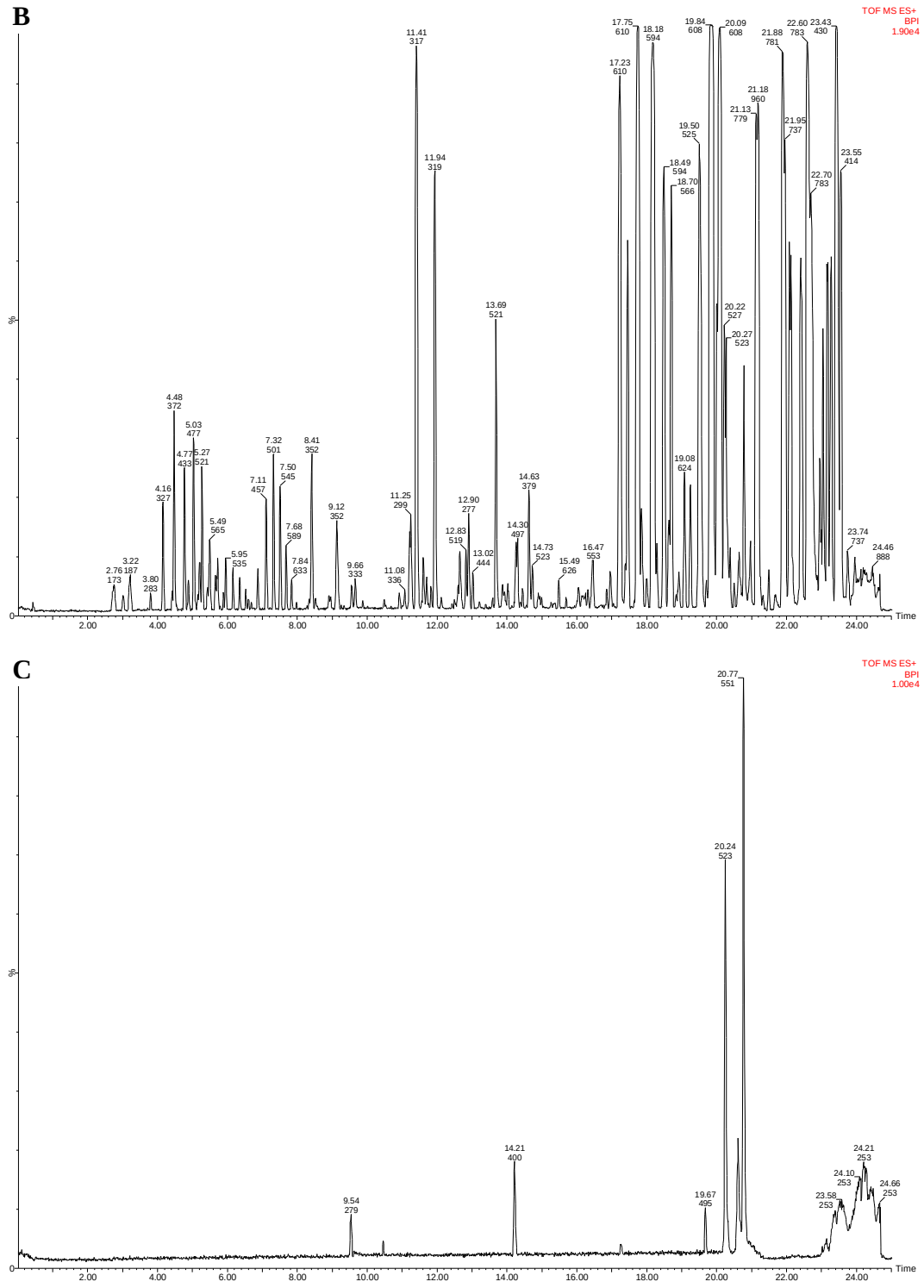


Figure 3.8: Base peak intensity chromatograms of the fruit (A), leaves (B) and stem (C) of *R. communis*.

Table 3.5: Retention time, mass to charge ratio and relative abundance of the peak of the fruit, leaves and bark of *O. sphaerocarpa*.

Retention time (minutes)	***m/z***	**Relative intensity (%)**		
		Fruit	**Leaves**	**Bark**
0.44	365			22
4.16	327	35	38	70
4.47	372	45	80	82
4.77/4.76	433	30	55	
5.02/5.04	477	52	100	88
5.26/5.27/5.28	521	32	82	80
5.48/5.49	565		30	45
5.70	491		20	30
5.94	535			25
7.09/7.10/7.11	457	32	30	65
7.30/7.31/7.32	501	45	42	78
7.49/7.50/7.51	545	32	32	65
7.66	589			30
14.01	567	75		
18.96	438			32
19.15	442			100
19.62/19.64	712	50		
19.74	442			58
20.19	523			48
20.29/20.30	523	60	80	
20.72/20.81	551	72	95	60
21.14	768	50		
22.31	533	48		
22.91	369	100		
23.27	412			40
23.39	410			88
23.55	414	52		62

Table 3.6: Retention time, mass to charge ratio and relative abundance of the peak of the fruit leaves and stem of *R. communis*.

Retention time (minutes)	***m/z***	**Relative intensity (%)**		
		Fruit	**Leaves**	**Stem**
4.15/4.16	327	30	20	
4.46/4.48	372	52	35	
4.77/4.75	433	48	25	
5.02/5.03	477	55	32	
5.24/5.27	521	48	25	
7.28/7.32	501	30	30	
7.50	545		22	
8.13	354	62		
8.41	352		28	
11.37/11.41	317		100	
11.94	319		75	
13.69	521		52	
17.23	610		92	
17.75	610		100	
18.18	594		98	
19.50	525		80	
19.84/20.09	608		100	
20.27/20.24	523	35	48	70
20.65	618	100		
20.76/20.77	551	52		100
21.18	960		88	
21.57	676	40		
21.88	781		98	
22.07	602	100		
22.60	783		100	
22.95	989	100		
23.43	430		100	

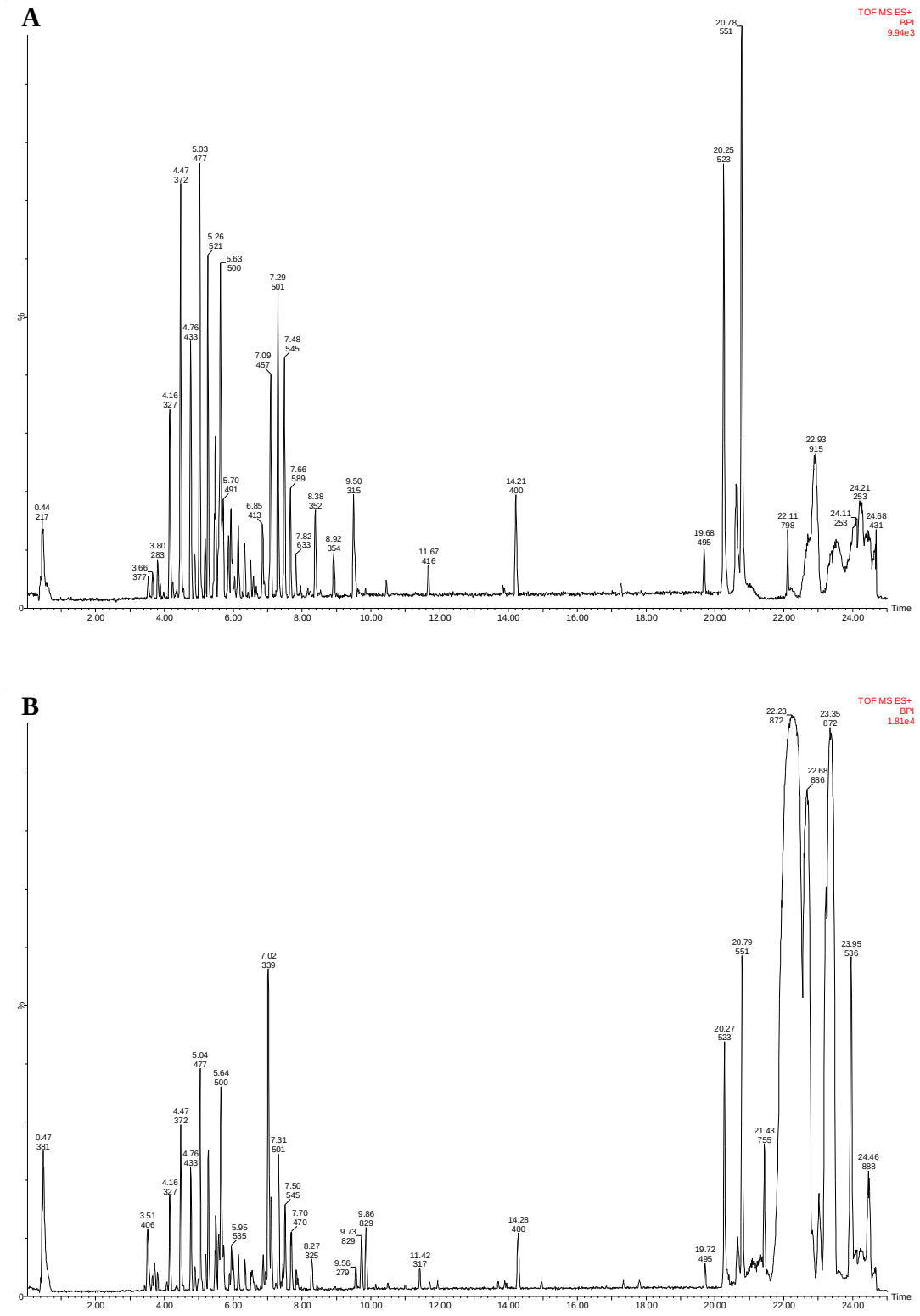


Figure 3.9: Base peak intensity chromatograms of the leaves (A) and root (B) of *H. acutatum*.

Table 3.7: Retention time, mass to charge ratio and relative abundance of the peak of the leaves and root of *H. acutatum*.

Retention time (minutes)	*m/z*	Relative intensity (%)	
		Leaves	Root
0.47	381		25
4.16	327	35	
4.47	372	72	30
4.76	433	45	22
5.03/5.04	477	78	40
5.26	521	62	
5.63/5.64	500	60	35
7.02	339		58
7.09	457	40	
7.29/7.31	501	55	25
7.48	545	42	
20.25/20.27	523	78	45
20.78/20.79	551	100	60
21.43	755		25
22.23	872		100
22.93	915	28	
23.35	872		100
23.95	536		58
24.46	888		22

The leaves and bark of *T. emetica* had different HPTLC chromatograms with the bark exhibiting more compounds than the leaves (Figure 3.1, track 9 and 10). *Trichilia emetica* has been reported to possess polyphenols such as caffeic acid, ferulic acid, gallic acid, chlorogenic acid and syringic acid (Germanó *et al*., 2006), as well as limonoids from *T. roka* (Nakatani *et al*., 1985), triterpenes from *T. pallida* (da Cunha *et al*., 2008) and tannins from the bark of *T. emetica* (Diallo *et al*., 2003). *T. emetica* also recorded a high content of phenolics in this study (Table 7.2).

The fruit and leaves of *D. cinerea* revealed similar HPTLC chromatographic profiles in terms of their major compounds (Figure 3.1, track 4 and 5); however the UPLC chromatograms revealed differences that had not been evident in the HPTLC chromatograms. The leaves showed greater complexity than the fruit, but there were a few similarities, for example, the peaks at Rt value 3.91, 4.14/4.15, 20.22/20.25 and 20.75/20.77 minutes were common in both plant parts, with approximately the same relative abundance (Figure 3.3). Major differences between the leaves and the fruit are the peaks at Rt value 7.09, 18.19, 22.62 and 23.44, which

were present in the leaves, but absent in the fruit. Peaks with Rt value 4.51 and 4.90 minutes were absent in the leaves and present in the fruit. Previous work by Joshi and Sharma (1974) isolated hentriacontanol, β-amyrin and sitosterol from the leaves of *D. cinerea*, and Rao *et al.* (2003) isolated (-)-mesquitol (2,3-*trans*-3',4'7,8-tetrahydroxyflavan-3-ol) and (-)-epicatechin. Tannins have also been isolated from this species (Banso and Adeyemo, 2007).

The leaves and bark of *S. cordatum* displayed similar compounds in the UPLC profile as well as in the HPTLC chromatogram. Similar compounds are at Rf value 0.1 (Figure 3.2, track 1 and 3.2, track 11), which were more visible in the bark as well as those with Rf value 0.65, 0.78 and 0.85, which were more visible in the leaves. The many similarities between these two plant parts are also confirmed by the UPLC chromatogram (Figure 3.4). The major peaks common among the two parts are those at Rt value of 4.47, 5.25/5.28, 7.07/7.12, 7.28/7.33, 7.47/7.52, 20.20/20.22 and 20.72/20.75 minutes. Several studies have been conducted on the composition of the *Syzygium* genus. Geyid *et al.* (2005) and Djoukeng *et al.* (2005) have reported the presence of polyphenols, tannins and unsaturated sterol/triterpens in the leaves of *S. guineense*; whilst Wojdylo *et al*., (2007) reported the presence of quercetin in *S. aromaticum*. Essential oils have also been isolated from the bud and leaf oils of *S. aromaticum*, such as eugenol and β-caryophyllenne (Srivastava *et al*., 2005). Wong and Lai (1996) also isolated some volatile compounds from several species of *Syzygium*, such as linalool, geraniol, myrcene and γ-terpinene. Veigas *et al.* (2007) and Benherlal and Arummughan (2007) both isolated anthocyanins from *S. cumini*, such as delphinidin, petunidin, malvidin and cyanidin. Other compounds that have been isolated from *S. formosanum* are caryophyllene oxide, friedelin, canophyllal, glutinol, alpha-terpineol, phytol, betulinic acid, uvaol, lupeol, betulin, ursolic acid, and oleanolic acid (Chang *et al*., 1999). Syzygiol has been isolated from *S. polycepphaloides* (Nishizawa *et al*., 1991). Candy *et al.* (1968) isolated friedelin, epi-friedelinol, β-sitosterol, gallic acid, ellagic acid and delphinidin from the bark, as well as cyanidin and delphinidin from the leaves of *S. cordatum*. It is evident that there are similarities in the composition of this genus, irrespective of where the plant grows, for example, friedelin, sitosterol and delphinidin which are present in almost all the species discussed above, yet they were isolated from species from different countries, such as Ethiopia, China, India, Malaysia and South Africa.

The leaves and bark of *B. salicina* exhibited similar compounds in their HPTLC chromatograms, though with higher intensity in the bark than in the leaves (Figure 3.2, track 2 and 3), for example compounds with Rf value 0.1, 0.35, 0.55 and 0.85. The leaves achieved a clearer separation than the bark where there was a bit of streaking. The two parts also displayed very similar UPLC profiles (Figure 3.5), but the leaves showed a greater complexity than the bark. Similar peaks were observed at Rt value 5.24/5.28, 6.10/6.19, 7.24/7.31, 20.20/20.23 and 20.72/20.76 minutes. The peak at Rt value 4.77 minutes was the most abundant in the leaves and that at 3.72 minutes was the most abundant in the bark. In both the leaves and the bark, there were both polar and non-polar compounds, with the polar ones having the lower Rt and Rf values (Figure 3.2 and 3.5). Neuwinger (1996) records that the plant contains alkaloids, triterpenes, saponins and polyphenols.

The fruit, leaves and bark of *O. sphaerocarpa* had very similar UPLC profiles (Figure 3.7). Although two unique peaks at retention times 14.01 and 22.91 minutes were observed in the fruit. The bark also had two peaks which distinguished it from the other plant parts, at 19.15 and 23.39 minutes. These peaks might be responsible for the varied activity of the fruit and may have contributed towards its toxicity (Table 6.1). The HPTLC chromatograms were also evidence of the differences between the three plant parts (Figure 3.2, track 7/8 and 9). The leaves and the bark had a more complex composition whereas the fruit, though simple in composition, had a unique band at Rf 0.75, which exhibited antimicrobial activity using the bioautography assay (Figure 4.10, track 5). Tirucallane triterpenes such as methyl 3α-hydroxy-tirucalla-8,24-dien-21-oate, methyl methyl 3α,24S-dihydroxytirucalla-8,25-dien-21-oate were isolated from the root of *O. insignis* (Liu and Abreu, 2006). The same group also isolated ozoroalide, which is a macrolide and anacardic acid methyl ester from the roots of *O. insignis* (Abreu and Liu, 2007). Ginkgoic and anacardic acid were also isolated from the twigs of *O. insignis* by Rea *et al*. (2003).

R. communis is one of the most studied plants due to ricin, a toxic alkaloid in the seed, (Balint 1974; Lombard *et al*., 2001); as well as the commercial application and use of castor oil which is used for constipation and in cosmetics. Lectins such as *R. communis* agglutinin have also been isolated from the plant (Kiyishe *et al*., 1974; Allen and Johnson, 1976; Hořejší, 1979; Roberts *et al*., 1985), as well as the enzymes pyridinium oxidase B (Fu and Robinson, 1972)

and pteridine oxidase (Hong 1980). Sitton and West (1975) reported the presence of an antifungal diterpene, caspene and mevalonic acid in the seedlings of *R. communis*. Ricinine has also been isolated from the leaves (Ferraz *et al*., 1999). The UPLC profiles of the plant parts were very different, especially the stem which contained very few compounds and due to its inactivity, it was not subjected to HPTLC separation. The HPTLC chromatograms of the fruit and the leaves were very similar (Figure 3.2, track 10 and 11) and so was the complexity of their UPLC profiles, with the leaves being more complex than the fruits (Figure 3.8).

The leaves and roots of *H. acutatum* had similar UPLC profiles, with similar peaks being recorded at Rt values 5.03/5.04, 5.63/5.64, 7.29/7.31, 20.25/20.27, 20.78/20.79 minutes. Helihumulone has been isolated as a bioactive compound from *H. cymosum*, with the essential oil from this species being reported to contain amongst others, α-pinene, limonene, 1,8-cineole, (Z)-β-ocimene and β-caryophyllenne (van Vuuren *et al*., 2006). Other studies, conducted as early as the seventies on this genus, have isolated different types of flavones, flavonols, flavonones, chalcones, quercetin and caryopyllen, terpenes and many other compounds (Bohlmann *et al*., 1978; Facino *et al*., 1990; Jakupovic *et al*., 1990; Pietta *et al*., 1991; Horie *et al*, 1995; Afolayan and Meyer, 1997; Wollenweber *et al*., 1998; Sala *et al*., 2003; Süzgec *et al*., 2005; Drewes *et al*., 2006; Drewes and van Vuuren, 2008; Lourens *et al*., 2008; Al-Rehaily *et al*., 2008; Ziaratnia *et al*., 2008). Dilika *et al*. (2000) isolated linoleic acid and oleic acid from *H. pedinculatum*, whilst Prinsloo and Meyer (2006) isolated β-amyrin and α-amyrin from *H. kraussii*. Arzanol, arzanol derivatives, helypyrone, rosifolial and tremetones have also been isolated from *H. italicum* subsp. *microphyllum* (Rosa *et al*., 2007). Numerous phytochemical anaylses have been performed on this genus in southern Africa and recently reviewed by Lourens *et al*. (2008).

The *P. meyeri* extract had a complex HPTLC chromatogram and this could be attributed to the fact that the whole plant was used in preparing this extract, resulting in a combination of the different compounds from the different plant parts. Most of the compounds were non-polar and visible as dark blue bands at the top of the chromatogram (Figure 3.1D, track 3). These bands were also visible under UV366 nm light, probably due to the presence of C=C double bonds in the compounds. The dark blue bands at the top of the chromatogram are typical of aromatic compounds whose presence is expected as this plant contains volatile compounds.

The leaves of *Z. mucronata* achieved separation in the HPTLC chromatogram (Figure 3.2, track 5); whilst the bark was dominated by many polar compounds which could not migrate through the stationary phase and therefore remained at bottom of the plate (Figure3.2, track 6). There were similarities though between the two chromatograms, for example, the compounds at Rf value 7.5 and 8.5 were common between the two extracts. Cyclopeptide alkaloids such as ziziphine N/O/P and Q have been isolated from the root of *Ziziphus oenoplia* var *brunoniana* (Susksamrarn *et al*., 2005).

The HPTLC chromatograms of the fruit, leaves and bark of *F. glumosa* were very different from each other (Figure 3.1, track 6, 7 and 8), with the fruit showing more complexity or achieving better separation. Most of the compounds in the bark and leaves were presumably polar and therefore did not migrate on the stationary phase, but remained at the bottom. Some of the compounds in the leaves which had not been visible after derivitization with vanillin were seen under UV366 nm light. Many compounds have been isolated from the *Ficus* genus and among these are lupeol, β-sitosterol, psoralene and bergapten which have been isolated from *F. pumila*, *F. sycomorus*, *F. carica*, *F. salicifolia*, *F. benjamina* and *F. aripuanensis* (Abu-Mustafa *et al*., 1964; Nascimento *et al*., 1999; Pistelli *et al*., 2000; Simo *et al*., 2008). Lopes *et al*. (1993) and Diaz *et al*. (1997) isolated moretenolactone and methoxyflavone from the leaves of *F. insipida*; whilst Herbert and Moody (1972) isolated antofine from the young trees of *F. septica*. Tuyen *et al*. (1998) isolated two new triterpenes from the leaf methanol extract of *F. fistulosa*, namely lanasterol-11-one acetate and 3β-acetyl ursa-14:15-ene-16-one. Other triterpenes that have been isolated from the leaves of *F. carica* include baueronol, calotropenyl acetate, methyl maslinate and oleanolic acid (Saeed and Sabir, 2002). Several other compounds have been isolated from the genus such as anthocyanins, amino acids, phenolic compounds such as gallic acid, chorogenic acid, epicatechin, syringic acid, tannins, saponins alkaloids, flavone aglycones, betulinic acid, platonic acid, catechin and rutin (Li and Kuo, 1998; Chiang and Kuo, 2003; Sandabe *et al*., 2006; Duenas *et al*., 2008; Simo *et al*., 2008; Veberic *et al*., 2008).

The leaves of *G. spatulifolia* exhibited a complex composition in the plant as previously reported in the genus. Several classes of compounds have been reported in this group of

plants. Reddy *et al*. (1975) reported the presence of such compounds as sitosterol, D-mannitol, gypsogenic acid methyl ester, haderagenin methyl ester, triterpenoids and oleanolic acid methyl ester from the wood and bark of *G. turgida*. Several other classes of triterpenoids have been isolated from various species of *Gardenia* such as *G. latifolifa, G. storckii, G. gordonii, G. jasminoides, G. coronaria* and *G. sootepensis* (Reddy *et al*., 1975; Davies *et al*, 1992; Zhao *et al*., 1994; Silva *et al*., 1997; Rukuchaisirikul *et al*., 1998). Inouye *et al*. (1974) and Wang *et al*. (2004) also isolated iridoid glycosides, geniposidic acid, gardenoside, shanzhiside, geniposide, genipin and gentiobioside. Flavans and flavonoids have also been isolated from species of *Gardenia* (Chhabra *et al*., 1977; Gunatilaka *et al*., 1982). The fruits of this genus have been reported to possess several compounds isolated from the gardenia yellow colour; such as genipin, crocin, crocetin and geniposide (Choi *et al*., 2001; Ozaki *et al*., 2002; Tsai *et al*., 2002). The UPLC chromatograms of *G. spatulifolia* are evidence of the many groups of compounds present within this plant (Figure 3.6). There were many similarities in the composition of the leaves and fruits, but the leaves had fewer compounds than the fruit. However, those compounds which were common to both two plant parts had a higher abundance in the leaves than the fruit, for example, compounds with Rt values of 4.15/4.16, 4.47/4.48, 5.02/5.03, 5.26/5.27 and 20.78/20.80 minutes, whose abundance was approximately 2 times greater in the leaves than the fruit. The fruit was found to be more toxic than the leaves to mammalian cells (Figure 6.1)) which could be due to some of the compounds present in the fruit that are absent in the leaves or a concentration thereof.

All the plant extracts studied in this section achieved good separation and displayed the diversity of compounds in them. These compounds are responsible for the *in vitro* activity found in the various plant extracts. This section needs further dedicated research in order to isolate and characterize the active compounds from the various plant parts, as they have the potential of being a source of a new antimalarial, antimicrobial or anti-oxidant agent.

CHAPTER 4: ANTIMICROBIAL ACTIVITY

4.1 Introduction

Micro-organisms are ubiquitous, i.e. they can be found in any environment that will support biological activity. They are found in soil, water, air, and particularly in association with plants, human beings and other animals. The indigenous flora of the human body is by far the most important source of micro-organisms that cause disease in humans. They are normally found in harmless close association with the human body surfaces and are often beneficial since they compete with potential pathogens for attachment sites and nutrients. However, under certain circumstances, they can cause infection. Micro-organisms can be divided into five groups, including bacteria, yeast, helminths, protozoa and viruses (Inglis, 1996; Bannister *et al*., 2000).

In developing countries, infectious diseases continue to be a major cause of mortality. The six biggest infectious killers are HIV/AIDS, malaria, tuberculosis, acute respiratory infections, diarrhoeal disease and vaccine-preventable diseases such as polio, measles and tetanus (Boutayeb, 2006). Bacterial infections are the most common micro-organisms responsible for opportunistic infections occurring in association with HIV/AIDS. These micro-flora facilitate the infection rate by the virus and significantly reduce the onset time of AIDS. Due to factors such as lack of patient compliance to antibiotic regimens, as well as indiscriminate use of these antibiotics, microbial strains have developed resistance to many of the available antibiotics (Geyid *et al*., 2005; Turkoglu *et al*., 2006).

4.1.1 Traditional medicine for infectious diseases

The traditional system of medicine mostly caters for infectious diseases such as diarrhoea, respiratory infections, sexually transmitted infections and skin problems. Plants, predominantly used in traditional remedies, are a rich source of secondary metabolites such as tannins, terpenoids, alkaloids and polyphenols and may possess antimicrobial activity (Geyid *et al*., 2005; Eloff and McGaw, 2006; Germanò *et al*., 2005; Cowan, 1999). Traditional healers use various plants and combinations thereof to manage different ailments such as diarrhoea, skin infections, toothache, elephantiasis and otitis media (Iwu, 1994). In Swaziland, about 63.4% of the population can access clinics or hospitals within an hour of

travel. The remaining percentage do not have this easy access and therefore have no choice but to consult traditional healers. To further worsen the situation, only 52.5% and 39.6% have access to safe water and adequate sanitation, respectively (Swaziland Ministry of Health, 2000.). This effectively means a great number of people are prone to contracting infections through poor sanitation and use of contaminated water.

Plants have long proven to be valuable sources of new drugs and ethnobotany should be utilized as a source of information for the research into efficacious plant metabolites, especially since the development of antibiotic resistant micro-organisms. Swazi traditional healers use many of the available plants, mostly indigenous to Swaziland, for the management of infectious diseases. These plants have been in use for many centuries and knowledge about them is passed from one generation to the next by word of mouth or through ancestral spirits. Traditional healers live in the communities with the people and are therefore easily accessible. Using traditional knowledge as a guide, the current study selected several species of micro-organisms in relation to the traditional use of the plants selected for screening.

4.1.2 Micro-organisms selected for this study

Two Gram-positive, two Gram-negative bacteria and a yeast were selected for this study. The selection of micro-organisms was based on the traditional use of the plant, as well as availability. The following were used, *Staphylococcus aureus (*ATCC 25923), *Staphylococcus epidermidis* (ATCC 2223), *Klebsiella pneumonia* (NCTC 9633), *Escherichia coli* (ATCC 25922) and *Candida albicans* (ATCC 10231).

4.1.2.1 The bacteria

Bacteria are single-cell organisms with a single circular DNA chromosome which is not enclosed in a nucleus as seen in eukaryotic cells. They also have specialized structures which serve different functions such as the pili, which are used for attachment to the host cell and the flagella used for movement (Figure 4.1).

To differentiate between Gram-positive and Gram-negative bacteria, the Gram reaction is used since it has the ability to bind to the cell wall teichoic acids found in Gram-positive cells and thereby resist discolouration by alcohol or acetone. This staining is important since Gram-

positive and Gram-negative organisms have different pathogenic potential and also different antibiotic susceptibilities (Bannister *et al*., 2000).

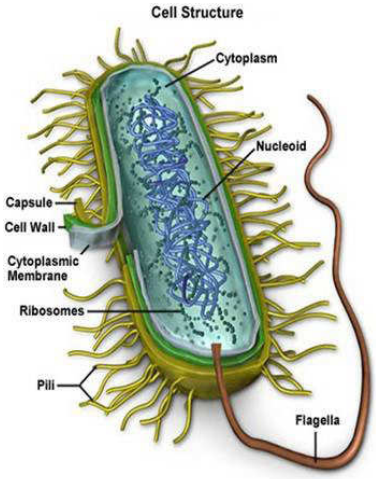


Figure 4.1 Structure of a gram-negative bacterium showing the different parts of its physiology (Era-net PathoGenoMics, 2006).

The cell wall of Gram-positive bacteria consists of the plasma membrane and a thick peptidoglycan layer, whilst that of Gram-negative bacteria consists of three layers, the plasma membrane, a thinner peptidoglycan layer and an outer membrane (Figure 4.2) (Pelczar *et al*., 1986). The composition of the cell walls of both Gram-positive and Gram-negative bacteria are important targets for antibiotics such as β-lactams and glycopeptides which act by inhibiting cell wall synthesis (Bannister *et al*., 2000). Cell wall damage of inhibition of synthesis may lead to cell lysis (Brooks *et al*., 2007). Apart from inhibiting of cell wall synthesis, drugs normally exhibit three other mechanisms of antimicrobial action:

- inhibition of cell membrane function (daptomycin),
- inhibition of protein synthesis (macrolides, chloramphenicol) and
- inhibition of nucleic acid synthesis (trimethoprim, sulfonamides) (Chambers and Deck, 2007).

This is dependent on the chemistry of the compounds involved, for example, detergents containing hydrophilic and lipophilic groups disrupt cytoplasmic membranes and kill the cell, such as nalidixin. Or they may specifically interfere with the biosynthetic functions of the cell membrane or act as ionophores, permitting rapid diffusion of cations through the cell membrane, thereby discharging the cell membrane. All these modes of antimicrobial action

are dependent on factors such as lipophilicity, hydrophilicity and pH of the environment to effectively inhibit microbial growth, one way or the other (Brooks *et al*., 2007).

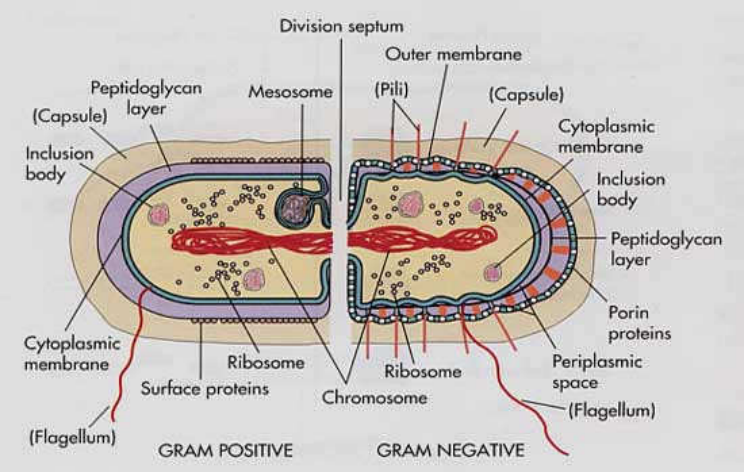


Figure 4.2: Structural differences between cell walls of Gram-negative and Gram-positive bacteria (Medical Microbiology, 2007).

4.1.2.1.1 *Staphylococcus aureus*

This is a Gram-positive bacterium (Figure 4.3) that is normally found on the skin, nasopharynx and intestines. It is mainly responsible for infections of the skin and mucosa such as impetigo, boils, abscesses, wound infections, postoperative infections, toxic shock syndrome, food poisoning and furunculosis (Humphreys, 2007). Infections occur when it enters the body through cuts and abrasions of skin and mucous membranes. They are characterized mainly by an intense inflammatory reaction and the production of pus or an abscess which makes it difficult to treat *S. aureus* infections, since the pathogen is 'walled-off' in the abscess (Brooks and Carroll, 2007). *Staphylococcus aureus* is a coagulase-positive bacteria and this is associated with its pathogenicity. The coagulase catalyses the conversion of fibrinogen to fibrin without the presence of thrombin. *Staphylococcus aureus* also expresses a fibronectin receptor on its surface which facilitates in its adhesion to host tissue and also produces extracellular enzymes such as hyaluronidase, collagenase and lipase which break down host tissue and facilitate invasion. Many clinical isolates (70-80%) of this pathogen produce penicillinase and are resistant to penicillins, for severe infections β-lactamase (or penicillinase)-resistant penicillins, cephalosporins or vancomycin are the drugs

of choice of treatment (Pelczar *et al*., 1986; Bannister *et al*., 2000; Kayser, 2005, Brooks and Carroll, 2007).

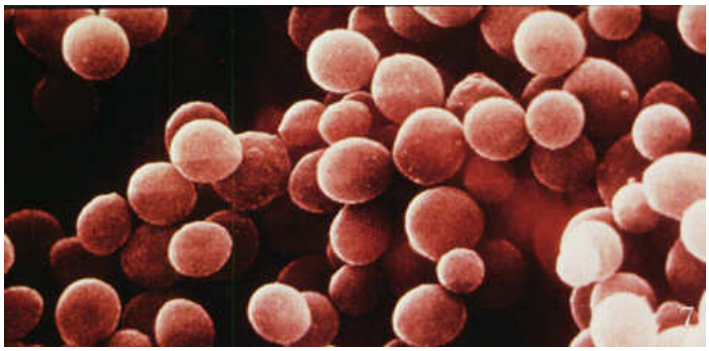


Figure 4.3: Electron micrograph of *S. aureus* (Todar, 2008).

4.1.2.1.2 *Staphylococcus epidermidis*

Unlike *S. aureus*, *Staphylococcus epidermidis* is a coagulase-negative bacterium (Figure 4.4). It colonizes the skin and mucuous membranes of the human body and requires a predisposed human skin to change into an infectious agent (Vuong and Otto, 2002). The most important group of infections caused by *S. epidermidis* are those on foreign bodies such as indwelling catheters, artificial joints, pacemakers, vascular grafts and cardiac valves and therefore responsible for a small percentage of wound infections, endocarditis and urinary tract infections (Pelczar *et al*., 1986; Kayser, 2005; Humphreys, 2007). These micro-organisms produce a biofilm which makes it difficult for many antibiotics to penetrate (Kayser, 2005). About 80% of *S. epidermidis* strains are resistant to methicillin and other antibiotics and vancomycin is the drug of choice in *S. epidermidis* infections. However, if there are vancomycin-resistant strains, rifampicin in combination with a glycopeptide is occasionally used (Humphreys, 2007).

4.1.2.1.3 *Klebsiella pneumoniae*

This Gram-negative pathogen is frequently associated with upper respiratory infections and can cause lobar pneumonia in patients with long-standing asthma, debilitating disease or

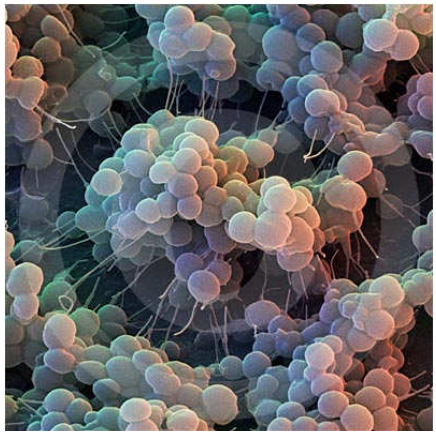


Figure 4.4: Electron micrograph of *S. epidermidis* (www.Scharf Photo.com, 2009).

alcoholism. It is characterized by copious amounts of sputum containing many neutrophils and large, capsulated Gram-negative rods. It is spread from the buccal and nasal discharges of infected individuals and in most instances, occurs as a secondary invader. *Klebsiella pneumoniae* (Figure 4.5) is also associated with otitis media, emphysema, pericarditis, meningitis and septicaemia. The organism is resistant to amoxicillin, ampicillin and other broad spectrum penicillins and is usually treated with cephalosporin and fluoroquinolones (Salle, 1973; Bannister *et al*., 2000; Chart, 2007).

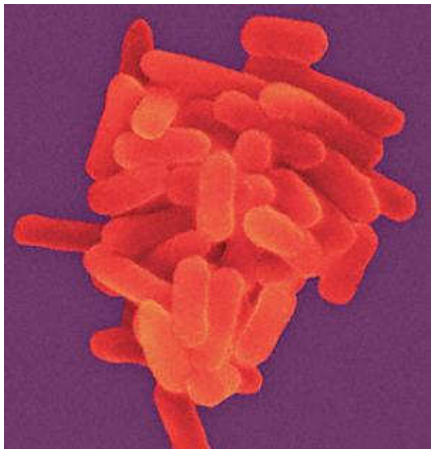


Figure 4.5: Electron micrograph of *K. pneumoniae* (KaiScience, 2007)

4.1.2.1.4 *Escherichia coli*

E. coli is a Gram-negative facultative anaerobe (Figure 4.6) and possesses capsular K antigens which facilitate adherence to host cells. They occur as normal flora in the lower portion of the gastrointestinal tract in humans and they are mainly responsible for gastroenteritis and urinary tract infections. Rarely, they may cause pulmonary infection, abscesses, skin and wound infections (Pelczar *et al*., 1986; Bannister *et al*., 2000). *E. coli* is susceptible to many antibacterial agents and therefore the urinary tract and septic infections may be treated using ampicillin, cephalosporins, tetracyclines, quinolines, aminoglycosides, trimethoprim and sulphonamides. Gastroenteritis is treated as most cases of diarrhoea with the use of fluid and electrolyte replacement, however, drug therapy can also be included through the use of doxycycline, trimethoprim and fluoroquinolones (Chart, 2007).

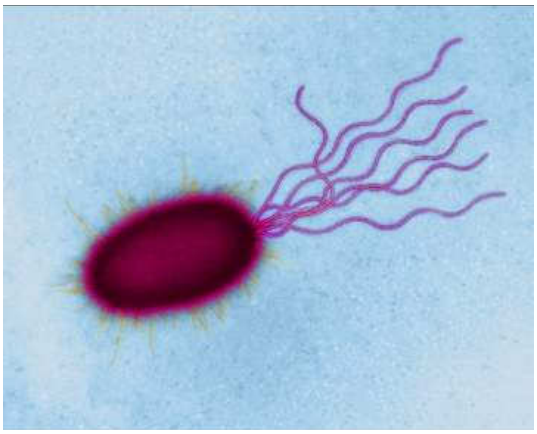


Figure 4.6: Electron Micrograph of *E. coli* (Astrographics.com, 2002).

4.1.2.2 *Candida albicans*

C. albicans is a yeast (Figure 4.7) that exists as normal flora of the mucous membranes and can become pathogenic, causing candidiasis of the mouth, vagina and alimentary tracts. Endocarditis, septicaemia and meningitis can also result from *C. albicans* infections. This yeast is an opportunistic pathogen and is prevalent in HIV/AIDS patients, where it causes significant morbidity among patients. Oral candidiasis is normally treated using topical antifungal agents such as nystatin, miconazole, fluconazole, itraconazole and amphotericin B. Vaginal candidiasis can be successfully treated with topical application of an imidazole, or with oral fluconazole or itraconazole. Due to the development of resistance in many *Candida* species, as well as the high toxicity of many available antifungal agents, the management of

Candida infections has become problematic (Peclzar *et al*., 1986; Eloff and McGaw, 2006; Runyoro *et al*., 2006; Warnock, 2007).

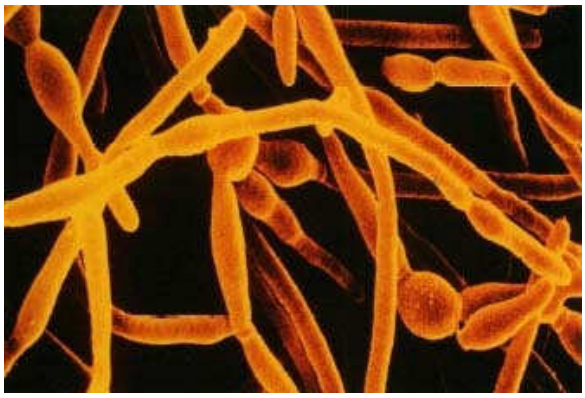


Figure 4.7: *Candida albicans* under electron microscopy (Phillips, 2007).

4.1.3 Antimicrobial drug screening

In screening for drugs with antimicrobial activity, one has to consider if the drug is selectively toxic towards the bacteria cell rather than the host cell (Brooks *et al*., 2007). Antimicrobial drug screening is essential to provide new and alternative drug treatments to the commonly used antibiotics as many bacterial strains develop resistance against them, such as the β-lactams. It is therefore the aim of this chapter to screen the plants for antimicrobial activity and also discuss the mammalian cell toxicity of the plant extracts (Chapter 6) in relation to the observed antimicrobial activity.

4.2 Materials and Methods

4.2.1 Micro-organism strains

The following reference bacterial and yeast strains were maintained in the Department of Pharmacy and Pharmacology (Pharmaceutical Microbiology Unit), University of the Witwatersrand. Two Gram-positive bacteria, namely *Staphylococcus aureus* (ATCC 25923) and *Staphylococcus epidermidis* (ATCC 2223); two Gram-negative bacteria, *Escherichia coli* (ATCC 25922) and *Klebsiella pneumoniae* (NCTC 9633) and one yeast *Candida albicans* (ATCC 10231) were kept at +4^{o}C.

4.2.2 Microplate dilution method

This method, described by Eloff (1998), is based on the use of a serial dilution technique using a 96-microwell plate for the determination of the minimum inhibitory concentration (MIC). The principle is to expose the plant extract at doubling dilutions to a known concentration of the test organism and thereafter determining growth using a tetrazolium-based indicator dye, *p*-iodonitrotetrazolium violet (INT). This dye is reduced to a red/pink colour by biologically active organisms (Yff *et al*., 2002; van Vuuren *et al*., 2006). The INT (initially colourless) acts as an electron acceptor and is reduced to coloured formazan crystals (pink/red) by metabolically active organisms (Eloff, 1998).

4.2.2.1 Preparation of extracts / positive controls

The plant extract (64 mg/ml) was prepared in acetone. Stock solutions of 0.01 mg/ml ciprofloxacin and 0.1 mg/ml amphotericin B (Sigma) were prepared and used as the positive controls for bacteria and yeast, respectively.

4.2.2.2 Culture maintenance and preparation

The cultures were maintained in Tryptone Soya broth (TSB; Oxoid) at +4 ^{o}C. TSB was prepared by dissolving 30 g in 750 ml sterile water and then sterilized by autoclaving the mixture at 121 ^{o}C for 15 minutes. Purity of cultures was determined by streaking the cultures on Tryptone Soya agar (TSA; Oxoid) plates and incubating them at 37 ^{o}C for 24 hours for the bacteria and 48 hours for the yeast. To prepare an overnight culture, the pure cultures were inoculated in TSB and incubated at 37 ^{o}C. To prepare approximately 1 x 10^{6} colony forming units (CFU)/ml inoculum, 1ml of culture was aseptically transferred into 100 ml sterile TSB.

4.2.2.3 Preparation of *p*-iodonitrotetrazolium violet

INT (Sigma) was prepared in sterile water to a stock concentration of 0.04% (w/v). To promote dissolution, the INT solution was put in a water bath (60 ^{o}C) for 30 minutes. Thereafter, it was stored at +4 ^{o}C and used within 48 hours.

4.2.2.4 Preparation of microtitre plates

Sterile water (100 µl) was pipetted into all the wells of a 96-well plate. Plant extract (100 µl) was added into the wells in the first row in duplicate (Figure 4.8). The extracts were serially diluted from row A to H in the plate by removing 100 µl from the first well into the next until

the last 100 µl in row H was discarded. An equal volume of culture (100 µl) was added into all the wells of the microtitre plate. The final concentration of extract in the wells ranged from 16 mg/ml to 0.125 mg/ml. After covering the plate with a sterile plastic seal, it was incubated for 24 hours (bacteria) and 48 hours (yeast) at 37 ^{o}C. Upon elapse of the incubation period, 40 µl INT was added and the plates left at room temperature for 6 hours (bacteria) or 24 hours (yeast). Thereafter, the plates were visualized and the MIC determined as the lowest concentration of extract where the red/pink colour was absent. For extracts showing high colour intensity interfering with visualization of MIC, the contents of the wells were streaked onto TSA to determine the minimum bactericidal concentration. Thereafter, the agar plates were incubated at 37 ^{o}C for 24 hours and 48 hours for the bacteria and yeast, respectively. The lowest concentration which inhibited the growth the microorganism was then considered the MIC. Ciprofloxacin and amphotericin B were used as bacterial and yeast controls, respectively; whilst acetone was used for 100% bacterial or yeast growth. In order to eliminate errors in the experiment, two columns were used per extract and the experiment was repeated three times.

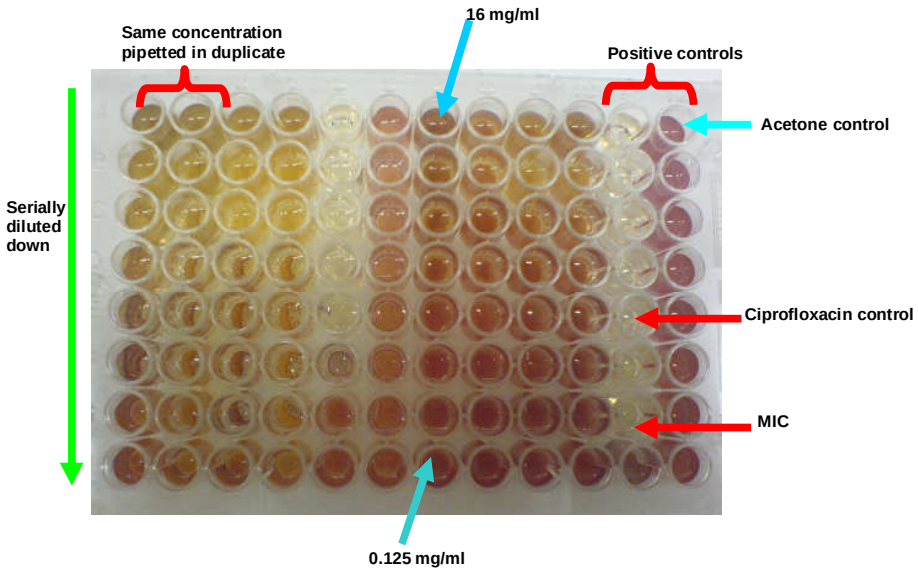


Figure 4.8: Plate layout for determining minimum inhibitory concentrations.

4.2.3 Bio-autographic assay using thin layer chromatography plates

The agar overlay method (Marston and Hostettmann, 1999) with *S. epidermidis* as the test organism was used to determine the antibacterial activity of the separated components in the

extracts. *Staphylococcus epidermidis* was chosen for bio-autography since it was the most susceptible pathogen to most of the extracts tested in this study (Table 4.1). Fourteen of the extracts, with MIC values less or equal to 1.0 mg/ml against *S. epidermidis* were identified for this assay. This assay employs the use of a separation technique, usually TLC to separate the components of the extract; thereafter, the test pathogen is either sprayed onto the chromatogram or an agar layer containing the test pathogen is applied on the chromatogram (Eloff and McGaw, 2006). The components of the extract move by diffusion from the stationary phase into the agar layer which contains the micro-organisms and if active inhibit the microbial growth. The agar plate is thereafter sprayed with the tetrazolim salt and inhibition of growth seen as white areas against a red background (Marston and Hostettmann, 1999).

4.2.3.1 Preparation of agar plates

Agar plates (20 m x 20 m) were prepared by pouring a sterile base layer of 150 ml TSA and leaving to set at room temperature.

4.2.3.2 Preparation of HPTLC plates

The high performance thin layer chromatography (HPTLC) plates were prepared as described in Section 3.2.1, using HPTLC silica gel 60 F_{254}, 20 x 10 mm aluminium plates (Merck). The HPTLC plates intended to be used for bio-autography were not derivatized with vanillin-sulphuric acid. The dried HPTLC plates were sterilized under ultraviolet light (254 nm) for 1 hour before being placed on the agar plates.

4.2.3.3 Preparation of HPTLC plates for bio-autography

The HPTLC plates were aseptically placed on the agar base and 150 ml of TSA containing approximately 1 x 10^6 CFU/ml *S. epidermidis* suspension (Section 4.2.2.2) poured as an overlay. The agar was allowed to set before being transferred to the fridge (+4 ^oC) for 2 hours to allow the compounds to pre-diffuse into the agar. Thereafter, the agar plates were incubated inverted at 37 ^oC for 24 hours. At the end of the incubation period, the plates were sprayed with 0.04% (w/v) INT to visualize the zones of inhibition.

4.2.4 Data analysis

The MIC results are presented as means of three independent experiments. The Student t-test was used to compare activity between the different plant parts within a species using Graphpad Prism® (version 4.0). A P value of less than 0.05 ($P<0.05$) was considered significant.

4.3 Results

4.3.1 Minimum inhibitory concentrations

An MIC value of 1.00 mg/ml was used as the cut-off point for *in vitro* activity, therefore, anything above this value was considered to have no significant activity (van Vuuren, 2008). Most of the extracts exhibited antimicrobial activity against at least one of the test organisms (Table 4.1). *Staphylococcus epidermidis* was the most susceptible bacteria to all the extracts tested with 45.5% of the plant extracts exhibiting inhibitory activity, followed by *S. aureus*, *K. pneumoniae* and *E. coli* (39.0%, 30.0% and 21.0%, respectively). Twenty (61.0%) of the extracts tested exhibited anti-*Candida* activity. The fruit of *O. sphaerocarpa* was the most active against all the pathogens tested and exhibited the lowest MIC value of 1.00 µg/ml against *S. epidermidis*, 20 µg/ml, against *S. aureus* and 70 µg/ml against *K. pneumoniae*. The bark of *F. glumosa* and the fruit of *D. cinerea* were the most active against *E. coli* with a MIC value of 0.25 mg/ml. *T. emetica* (leaves), *L. intermedia* (flower), *R. communis* (leaves) and *Z. mucronata* (leaves) were the most active against *C. albicans* with MIC values of 0.22, 0.25, 0.29 and 0.23 mg/ml, respectively. The fruit of *G. spatulifolia* was the least active of all the extracts with an MIC value greater than 5 mg/ml for all the pathogens. The root of *H. acutatum* was the least active against the Gram-positive micro-organisms, *S. aureus* and *S. epidermidis* with an MIC value greater than 12 mg/ml. The inhibition of growth of the test organisms when exposed to the control antimicrobial confirmed the susceptibility of the organism. When comparing the activity of the various plant parts, it was found that overall, the leaves were the most potent with an average MIC value of 1.69 mg/ml of all the pathogens, whereas the stem, root or stem/root was the least active with an average MIC value of 3.18 mg/ml. The bark and the flower/fruit had similar activity, MIC value of 2.20 and 2.74 mg/ml, respectively.

4.3.2 Bio-autographic assay

The bio-autography results showed activity for most of the extracts tested (Figure 4.9 and 4.10). This confirms the presence of antimicrobial constituents within the extracts as all the extracts subjected to bio-autography were active against *S. epidermidis* using the MIC assay (Table 4.1). Of note is the fact that the active constituents, shown where there are clear zones with no bacterial growth, correspond to those compounds that are UV active, that is they have double bonded conjugated systems. This is true of terpenoids, steroids, nucleic acids, and other compounds which possess conjugated double bonds. The fruit of *O. sphaerocarpa* had the biggest zone of inhibition (Figure 4.9) which confirms the activity that was observed in the MIC assay, with a MIC value of 1.00 μg/ml against *S. epidermidis*. The leaves and bark of *O. sphaerocarpa* and *T. emetica*, as well as the leaves of *S. cordatum* all showed good activity in the bio-autographic assay and MIC assay.

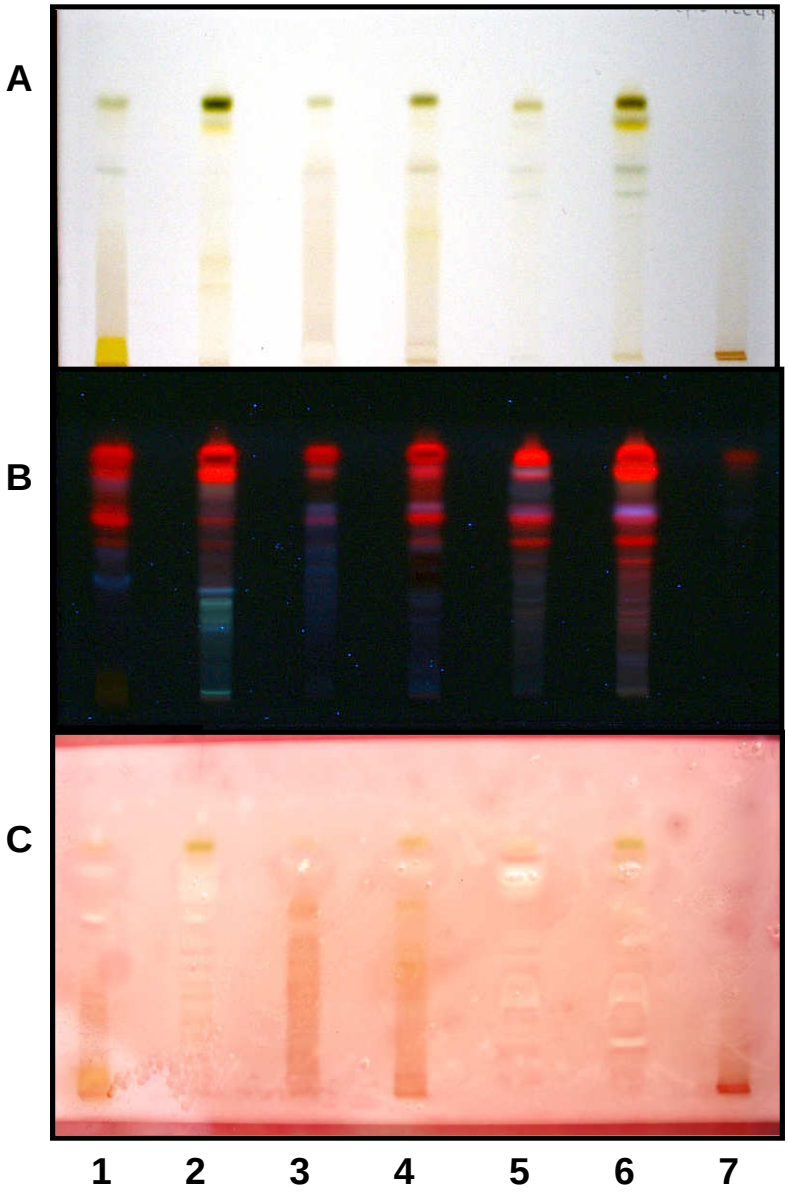
4.4 Discussion

In this study, 23 out of the 33 extracts from the 15 species investigated were found to have some *in vitro* activity against at least one of the micro-organisms tested. The extracts were more active against the Gram-positive bacteria compared to the Gram-negative bacteria (Table 4.1). This is in agreement with studies conducted by other researchers (Lall and Meyer 2000; Mathabe *et al*., 2006; Kamatou, 2006) and could be attributed in part to the fact that the cell walls of Gram-negative bacteria are chemically and structurally more complex than those of Gram-positive bacteria (Figure 4.2). The differences in the amount of peptidoglycan, presence of receptors in the lipids and the nature of cross-linking activity or autolytic enzymes determines the penetration, binding and activity of the drugs (Brooks *et al*., 2007) and thus may render Gram-negative bacteria more difficult to permeate by the extract.

O. sphaerocarpa was active against most of the pathogens tested (Table 4.1). The fruit, which has no recorded medicinal use, was very active against the Gram-positive bacteria (MIC value ranging from 0.001-0.02 mg/ml). This activity may also be due in part to the seeds which were not separated from the flesh during extraction as the whole fruit is traditionally used (PP Ndlovu, personal communication). It has been reported that certain seeds contain toxic compounds, for example, *R. communis* has been reported to contain a very toxic compound (ricin) in its seed which is not found in the other plant parts, such as the leaves or the stem

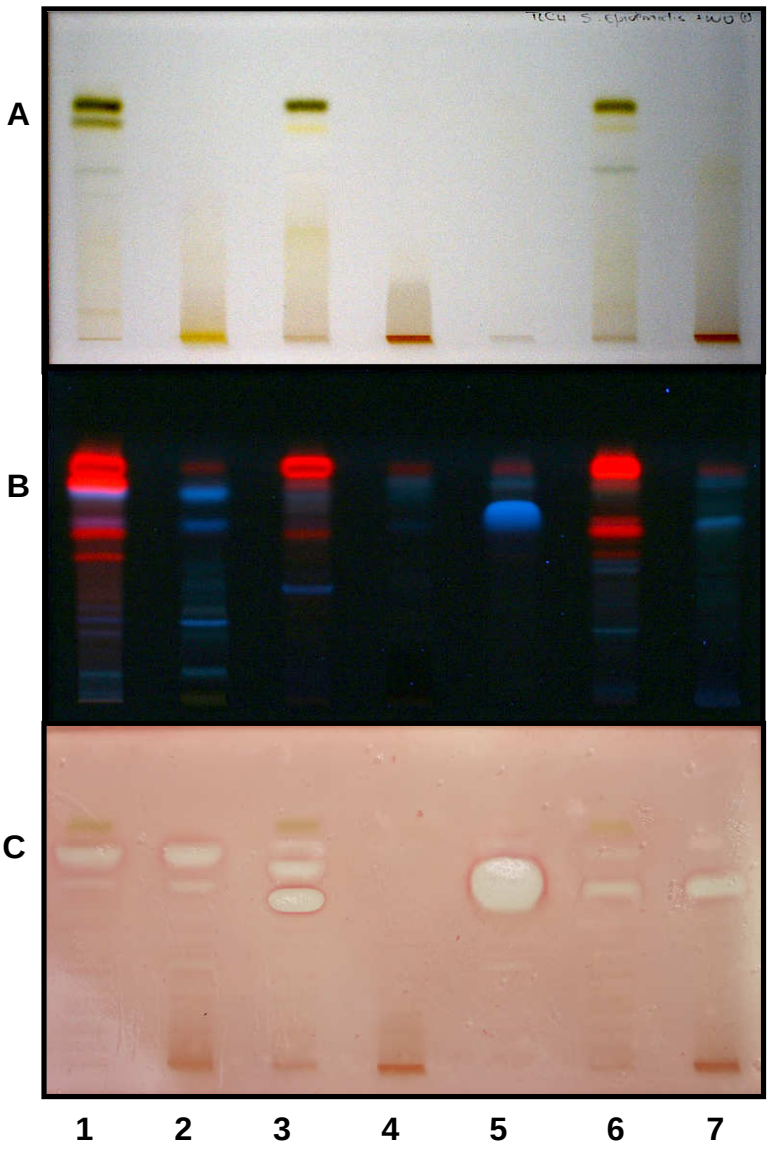
Table 4.1: *In vitro* antimicrobial activity (MIC, mg/ml) of the traditionally used medicinal plant extracts from Swaziland.

Plant species and controls	Plant part	Antimicrobial MIC (mg/ml)				
		***S. epidermidis* (ATCC 2223)**	***S. aureus* (ATCC 25923)**	***K. pneumoniae* (NCTC 9633)**	***E. coli* (ATCC 25922)**	***C. albicans* (ATCC 10231)**
Berkheya setifera	flower	4.00	4.00	3.33	3.00	2.00
	stem/root	8.00	4.00	1.33	2.00	1.33
	Leaves	1.67	3.33	1.33	5.33	0.67
Breoanadia salicina	Leaves	1.33	5.33	2.67	1.33	2.67
	Bark	4.00	8.00	2.67	11.00	5.33
Dichrostachys cinerea	Fruit	0.38	0.29	0.56	0.25	0.67
	Leaves	0.42	0.44	0.67	0.50	0.42
Ficus glumosa	Fruit	1.50	3.00	2.00	2.00	2.00
	Leaves	2.00	4.00	1.33	2.00	3.33
	Bark	0.25	0.25	5.33	0.25	5.33
Gardenia spatulifolia	Fruit	6.00	12.00	8.00	8.00	12.00
	Leaves	1.67	4.00	1.33	2.00	0.39
Guilleminea densa	whole plant	1.25	4.00	1.33	1.33	1.00
Helichrysum acutatum	Leaves	1.67	4.00	1.33	2.67	0.50
	Roots	13.33	>16	2.00	2.67	4.00
Leonotis intermedia	flower	3.00	4.00	1.00	3.00	0.25
	Stem	3.00	4.00	1.50	1.50	3.00
Ozoroa sphaerocarpa	Fruit	0.001	0.02	0.07	4.00	0.58
	Leaves	0.67	2.67	0.50	2.67	1.00
	Bark	0.04	1.00	0.67	1.00	1.00
Priva meyeri	whole plant	0.83	0.50	1.33	0.50	0.67
Ricinus communis	Fruit	1.00	0.67	1.33	1.33	0.50
	Leaves	0.83	0.66	3.33	1.33	0.29
	Stem	1.50	2.00	1.00	1.50	0.50
Syzygium cordatum	Leaves	0.04	5.00	0.44	3.00	1.00
	Bark	0.38	2.00	0.25	1.50	1.50
Terminalia. phanerophlebia	Leaves	0.33	0.33	0.67	1.33	0.77
	Bark	1.50	1.00	1.33	1.00	0.75
Trichilia emetica	Leaves	0.16	0.19	2.00	0.83	0.22
	Bark	0.07	0.58	2.67	6.67	0.78
Ziziphus mucronata	Fruit	1.33	3.33	1.33	4.00	4.00
	Leaves	2.00	4.00	1.50	3.00	0.23
	Bark	1.00	0.50	1.33	2.00	4.00
Controls (a: Ciprofloxacin; b: Amphotericin B)		3.9×10^{-5}[a]	5.5×10^{-4}[a]	9.1×10^{-5}[a]	4.4×10^{-4}[a]	0.016[b]



Track name	Plant sample
1	*T. phanerophlebia* leaves
2	*P. meyeri* whole plant
3	*D. cinerea* fruit
4	*D. cinerea* leaves
5	*R. communis* fruit
6	*R. communis* leaves
7	*F. glumosa* bark

Figure 4.9: Thin layer chromatography plates (A) of seven of the extracts showing visualization under; (A) 356 nm (B) 254 nm (C) inhibition zones of extracts on *S. epidermidis*.



Track name	Plant sample
1	*T. emetica* leaves
2	*T. emetica* bark
3	*S. cordatum* leaves
4	*S. cordatum* bark
5	*O. sphaerocarpa* fruit,
6	*O. sphaerocarpa* leaves
7	*O. sphaerocarpa* bark

Figure 4.10: Thin layer chromatography plates (B) of seven of the extracts showing visualization under; (A) 356 nm (B) 254 nm (C) inhibition zones of extracts on *S. epidermidis*.

(Ferraz *et al*., 999). The fruit of *O. sphaerocarpa* also displayed toxicity to human kidney epithelial cells (IC_{50} value of 10.39 ± 1.05 μg/ml; Table 6.1). This may indicate general

toxicity without specificity in the plant. A further study where the flesh and seeds are separated and re-tested for antimicrobial activity and toxicity, or using bio-autography to separate the active constituent and re-testing for activity, can determine if the activity in the

fruit is due to its general toxicity or the presence of an active constituent responsible for antimicrobial activity. The bark and leaves also showed good activity against all the pathogens and they have been reported to be traditionally used for the treatment of diarrhoea by Adeniji *et al*. (2000). *O. insignis*, a close relative of *O. sphaerocarpa*, is also traditionally used by the Venda people of South Africa for the treatment of diarrhoea (Mathabe *et al*., 2006).

In a study by Mathabe *et al*. (2006), the stem bark methanolic extract was found to have *in vitro* antibacterial activity with MIC values as low as 0.156 and 0.078 mg/ml for *S. aureus* and *E. coli*, respectively. Runyoro *et al*. (2006) studied the anti-*Candida* activity of *O. insignis* using the bio-autography agar overlay method and found no activity using the root/bark methanolic extract. In this study, the anti-*Candida* activity for the fruit and bark extracts were 0.58 and 1.00 mg/ml, respectively. The differences in anti-*Candida* activity observed may be due to the different methods used; with the different compounds in the extract acting synergistically to produce the overall activity observed in the MIC method. The bio-autography results show that the three plant parts contain different compounds, which are responsible for inhibition of growth of *S. epidermidis* in this plant (Figure 4.10C; track 5/6 and 7). It seems that the active compound in the fruit and bark (Rf 7.8) may be the same or very similar; alternately that the fruit contains more of that compound resulting in the higher activity observed. This very favourable result warrants further investigation with the future development of this plant for medicinal purposes. This can be an advantage, especially during its fruiting season, as it might reduce the unsustainable harvesting of the bark material which results in the death of the plant.

D. cinerea (fruit and leaves) inhibited the growth of all the pathogens tested in this study. These results support their use for skin infections, bronchitis and toothache (Watt and Breyer-Brandwijk, 1962; Kambizi and Afolayan, 2001; Venter and Venter, 2002; Koné *et al*., 2004). The results in this study are in agreement with those of Eisa *et al*. (2000) who reported antibacterial activity of the methanol extract of the fruit and leaf against *Bacillus subtilis, S. aureus* with MIC values of 1.30, 2.60 and 1.50, 3.05 mg/ml, respectively. In contrast, in this study, it was found that there was antifungal activity against *C. albicans,* whereas there was no activity in the study by Runyoro *et al*. (2006) and Steenkamp *et al*. (2007). Bio-autography

results (Figure 4.9C; track 3 and 4) show that the same compound is responsible for the antimicrobial activity in this plant, with the compound present in higher concentrations in the leaves than the fruit. Banso and Adeyemo (2007) investigated the antimicrobial properties of tannins isolated from the root of *D. cinerea*. It is interesting to observe that these tannins had MIC values ranging from 4.0 to 5.5 mg/ml against *S. aureus, E. coli, Pseudomonas aeruginosa*, *Shigella boydii* and *Shigella flexneri*, which were higher than those obtained in this study for the leaves and fruit (MIC values less than 1.0 mg/ml; Table 4.1). This further demonstrates that the different compounds present in an extract act synergistically to produce the overall effect observed using the MIC method.

The leaves and bark of *T. emetica* were mostly active against the Gram-positive bacteria as well as *C. albicans*, with MIC values ranging from 0.07 – 0.78 mg/ml (Table 4.1). A similar study conducted by Geyid *et al.* (2005), showed the inhibitory activity of the fruit against *S. aureus* (ATCC 27853) and *C. albicans* (clinical isolate) at concentrations as low at 0.5 mg/ml. The ethyl ether extract of the root of *T. emetica* was found to have good activity against a clinical isolate of *S. aureus* (MIC value of 0.031 mg/ml) in a study by Germanò *et al.* (2005). Shai *et al.* (2008) reported antimicrobial activity of the leaf extracts of the *T. emetica* which had MIC values between 0.3 and 2.5 mg/ml against *S. aureus*, *E.coli* and *C. albicans*. These results are in agreement with the results of the current study and confirm the antimicrobial activity of this plant, which further supports its traditional use for the treatment of bacterial infections such as urethral discharge and skin diseases (Watt and Breyer-Brandwijk, 1962; Adeniji *et al.*, 2000; van Wyk *et al.*, 2000; Venter and Venter, 2002). It is possible that limnoids, which have been isolated from the root bark of *T. emetica* (Nakatani *et al.*, 1985), are responsible for the antibacterial activity found in this plant. In Amusan *et al.* (2007) the presence of alkaloids, flavonoids, glycosides, polyphenols and steroids in the stem bark of this plant were reported, which correlate to the results of this study, where a high content of flavonoids (26.40 ± 0.72 mg RE/g extract) and appreciable phenolics (17.00 ± 1.89 mg GAE/g extract) have been reported (Table 7.2). Bio-autography results (Figure 4.10C track 1 and 2) have shown that two major compounds are responsible for the antimicrobial activity of the plant and are present in both the leaves and bark. The two compounds have the same Rf values and it is possible that they are one and the same compound.

S. cordatum (leaves and bark) are used as an emetic, for stomach troubles and to treat respiratory ailments such as tuberculosis (Venter and Venter, 2002; van Wyk *et al.*, 2000). These extracts were the most active against *S. epidermidis* and *K. pneumoniae* (Table 4.1) and the latter activity supports its traditional use in the treatment of respiratory ailments such as pneumonia. Mathabe *et al.* (2006) conducted a similar experiment and also found similar activity in the bark methanolic extract against *S. aureus* (ATCC 25923) and *E. coli* (ATCC 35218), MIC value of 0.312 mg/ml for both pathogens. The bark extract had no appreciable anti-*Candida* activity in our study (MIC value of 1.5 mg/ml), which was similar to that obtained by Steenkamp *et al*., (2007) who reported an MIC value of 3.75 mg/ml. The bark methanolic extract was also found to have activity against *S. aureus*, *E. coli* and *K. pneumoniae* (Samie *et al*., (2005). The antimicrobial activity of the essential oil of *S. aromaticum* has also been reported against *S. aureus*, *E. coli*, *K. pnuemoniae* and *C. albicans* using the disc diffusion assay (Dorman and Deans, 2000) and MIC method (Hammer *et al*., 1999). The bio-autography results (Figure 4.10C track 3 and 4) showed three major compounds in the leaves that were responsible for inhibiting the growth of *S. epidermidis*. These compounds were absent in the bark. The MIC results (Table 4.1) showed a ten times greater activity of the leaves compared to the bark against *S. epidermidis*.

Z. mucronata was among the most active against *C. albicans*, together with *L. intermedia* and *R. communis* (Table 4.1). The roots of *Z. mucronata* have previously been reported to possess compounds with anti-*Candida* activity using the bio-autography assay (Runyoro *et al*., 2006). A close relative, *Z. nummularia* (aerial parts) have also been reported to possess antimicrobial activity using the disc diffusion assay (Mahasneh, 2002). These results are exciting and need to be explored further, to possibly isolate the active constituents which could assist in the treatment of these pathogens. The aerial parts of *L. leonorus* have been reported to possess antibacterial activity against *S. auerus*, *E. coli*, *B. cereus* and *K. pneumoniae* with MIC values between 2.0 and 3.0 mg/ml (Kamatou, 2006). Steenkamp *et al*. (2004) also reported MIC values greater than 4.0 mg/ml for *L. leonorus*. These results are in agreement with the current study where MIC values between 1.0 and 4.0 mg/ml are reported (Table 4.1) for both the flower and stem extracts. All the parts of *R. communis* tested in this study exhibited anti-*Candida* activity with MIC values ranging between 0.25 and 0.59 mg/ml. Sitton and West (1975) reported the presence of an antifungal phytoalexin, casbene, in the seedlings of *R.*

communis. It is possible that this phytoalexin is responsible for the growth inhibition of *C. albicans* displayed in this study.

Only the bark of *F. glumosa* displayed significant antimicrobial activity (Table 4.1). The fruit and leaves had MIC values greater than 1.0 mg/ml against all the pathogens tested. The bark bio-autography results did not show any inhibition of growth against *S. epidermidis* (Figure 4.9C; track 7), suggesting that the activity observed with the MIC assay as due to synergistic interactions between the different compounds in the bark. Steenkamp *et al*. (2007) reported no activity of the fruit methanolic and aqueous extract of both *F. capensis* and *F. sycomorus* against *C. albicans*. The results of this study also displayed very low activity of all the extracts (MIC values greater than 1.0 mg/ml). The leaf methanolic extract of *F. thonningii* had MIC values ranging from 0.094 to 0.75 mg/ml against an aminoside-resistant and -sensitive strain of *Enterococcus faecalis* and 0.023 to 0.375 mg/ml against *Streptococcus pyogens* in a study by Koné *et al*. (2004). Annan and Houghton (2008) have reported the antimicrobial activity of the aqueous extract of *F. asperifolia* (MIC value of 0.128 mg/ml) against both *S. aureus* and *E. coli*. These results are similar to what is reported in this study for the bark methanolic extract (MIC value of 0.25 mg/ml; Table 4.1) for both pathogens.

The leaves and bark of *T. phanerophlebia* displayed different inhibitory profiles to the different pathogens in this study, with the leaves having greater inhibition, especially on the Gram-positive bacteria *S. aureus* and *S. epidermidis* (Table 4.1). Steenkamp *et al*. (2004) also reports the presence of antimicrobial activity in the methanolic and aqueous extracts of *T. sericea* with a MIC value of 1.0 mg/ml for both *S. aureus* and *S. pyogens*, both Gram-positive and lack of activity against the Gram-negative species used in their study. They further report the anti-*Candida* activity of the root methanolic and aqueous extract of *S. sericea*, with MIC values of 2.5 and 2.0 mg/ml, respectively (Steenkamp *et al*., 2007). Masoko *et al*. (2005) also reported the anti-*Candida* activity of methanolic extracts of six species of *Terminalia*, with MIC values ranging from 0.1 to 0.64 mg/ml, which is similar to that reported by Shai *et al*. (2008) for the leaf dichloromethane extract of *T. phanerophlebia* and *T. sambesiaca*. The same study also reported antibacterial activity of the extract of *T. phanerophlebia* against *S. aureus* and *E. coli*, with MIC values of 0.3 mg/ml for both pathogens. Tshikalange *et al*. (2005) previously reported on the very weak activity in the aqueous extract of *T. sericea*

against four Gram-positive pathogens (MIC values ranged from 1.0 to 20.0 mg/ml). The bio-autography results also confirm the presence of compounds with antimicrobial activity in the leaves (Figure 4.9C; track 1).

The leaves of *H. acutatum* displayed better antimicrobial activity (MIC values ranging from 0.5 to 4.0 mg/ml) compared to the roots with MIC values ranging between 2.0 to greater than 16.0 mg/ml for all the pathogens tested. The roots were most ineffective against the Gram-positive micro-organisms compared to the Gram-negative bacteria or yeast. In the review by Lourens *et al*. (2008) on the biological activities of South African *Helichrysum* species, several species were recorded to possess antimicrobial activity with MIC values less or equal to 1 mg/ml such as *H. appendiculatum*, *H. argyrosphaerum*, *H. aureonitens*, *H. bellum*, *H. cymosum*, *H. nudifolium*, *H. trilineatum* and *H. tomentosulum*. Steenkamp *et al*. (2004) also reported poor activity of the leaf methanolic extract of *H. foetidum*, which had MIC values greater than 4 mg/ml against *S. aureus*, *E. coli*, *S. pyogens*, and *P. aeruginosa*, whilst van Vuuren *et al*. (2006) reported good activity from the acetone extract of *H. cymosum* with MIC values ranging from 0.078 to 0.313 mg/ml for a variety of Gram-positive and Gram-negative bacteria and yeasts. Using bio-autography and liquid chromatography, the latter authors were able to isolate the active compound from the extract and subsequently determine its antimicrobial activity. The active compound was identified as helihumulone and displayed activity against the pathogens tested, with MIC values ranging from 0.031 to 0.125 mg/ml. Following a literature survey, it would appear that this is the first report on the antimicrobial activity of *H. acutatum*. The leaves of *H. acutatum* were not toxic to human kidney epithelial cells nor caused lysis of red blood cells (Table 5.1 and 6.1). However they displayed some activity against *C. albicans*, which could mean there is a specific antimicrobial target resulting in the inhibitory effect on the yeast cells. The leaves also had a high content of phenolics (182.80 ± 4.65 mg GAE/g extract; Table 7.2) and these could possibly account for the inhibitory effect on yeast cells. This warrants further investigation into the mechanism of action leading to cell death.

P. meyeri has previously not been investigated for antimicrobial activity, but it exhibited both antibacterial and anti-*Candida* activity in the current study (Table 4.1). The bio-autography results do not display much inhibitory effect on the growth of *S. epidermidis*, but several bands

visibly inhibited microbial growth (Figure 4.9C; track 2). *Lippia javanica*, which is from the same family as *P. meyeri* has been reported to possess both the antibacteterial and antifungal properties using the time-kill methodology (Viljoen *et al.*, 2005).

The fruit and leaves of *G. spatulifolia* did not possess favourable antimicrobial activity except for the leaves which had anti-*Candida* activity (MIC value of 0.39 mg/ml). Magassouba *et al.* (2007) reported activity in the root bark methanolic extract of *G. ternifolia* against *S. aureus* (MIC value of 0.0625 mg/ml) and *C. albicans* (MIC value of 0.25 mg/ml). The activity against *C. albicans* was similar to what was reported in the current study. Anti-HIV properties for a flavone and a coronalolide methyl ester isolated from the twigs and leaves of *G. tubifera* have been reported (Reutrakul *et al*., 2004).

G. densa, *B. setifera* and *B. salicina* did not display any antimicrobial activity against the tested pathogens (Table 4.1). A literature survey did not reveal any data on the antimicrobial activity or testing of the plants, however a relative of *B. setifera*; *B. speciosa* is reported to possess inhibitory activity towards *Schistosoma haematobium* (Sparg *et al*., 2000).

In conclusion, this chapter was able to evaluate the antimicrobial potential of many of the species studied, some of which were being documented for the first time, such as *B. setifera*, *H. acutatum*, *O. sphaerocarpa* and *P. meyeri*. The findings of this chapter support the traditional use of these species for the treatment of microbial infectious diseases.

CHAPER 5: ANTIMALARIAL ACTIVITY

5.1 Introduction

5.1.1 Malaria worldwide

Malaria is a major health problem in many parts of the world, particularly Africa, South America and south-east Asia; with an incidence of almost 300 million clinical cases and over one million deaths per year (WHO, 2008). Almost 90% of these deaths occur is sub-Saharan Africa where young children and pregnant women are the most affected (Ruxin *et al*., 2005). In children, cerebral malaria, severe anaemia and respiratory distress due to systemic acidosis are the major causes of death. Adult death frequently results from renal failure or acute respiratory distress syndrome, with local lung pathology. Pregnant women and newborns are also affected by malaria and up to 200 000 newborns die as a consequence of malaria each year (Steketee *et al*., 2001). The most common symptoms of malaria are fever, rigors, headache, sweating, tiredness, myalgia, abdominal pain, diarrhoea, loss of appetite, nausea, vomiting, cough and "flu-like" symptoms (National Department of Health, 2008b).

5.1.2 Malaria in South Africa and Swaziland

Malaria is endemic in almost all countries in sub-Saharan Africa with the exception of Lesotho (Figure 5.1). Geographical distribution varies within each country and there are also seasonal disease peaks. In South Africa these peaks are from October to May in low altitude areas (below 1000m), such as parts of Mpumalanga, Limpompo and North Eastern KwaZulu-Natal (National Department of Health, 2008a). Limited transmission may occur in the North West and Northern Cape provinces along the Malopo and Orange rivers (Figure 5.1). Whereas in Swaziland the peak transmission period is from December to May and mainly occurs in the Lubombo region (51%), followed by Hhohho (34%), Manzini (9%) and Shiselweni (6%) (National Malaria Control Program, 2004/2005).

The disease is seasonal and unstable occurring mainly during or after the rainy season. In Swaziland, it is estimated that about 30% of the population is at risk of malaria and that it accounts for 0.9% of all mortality in the country (National Malaria Control Program, 2004/2005).

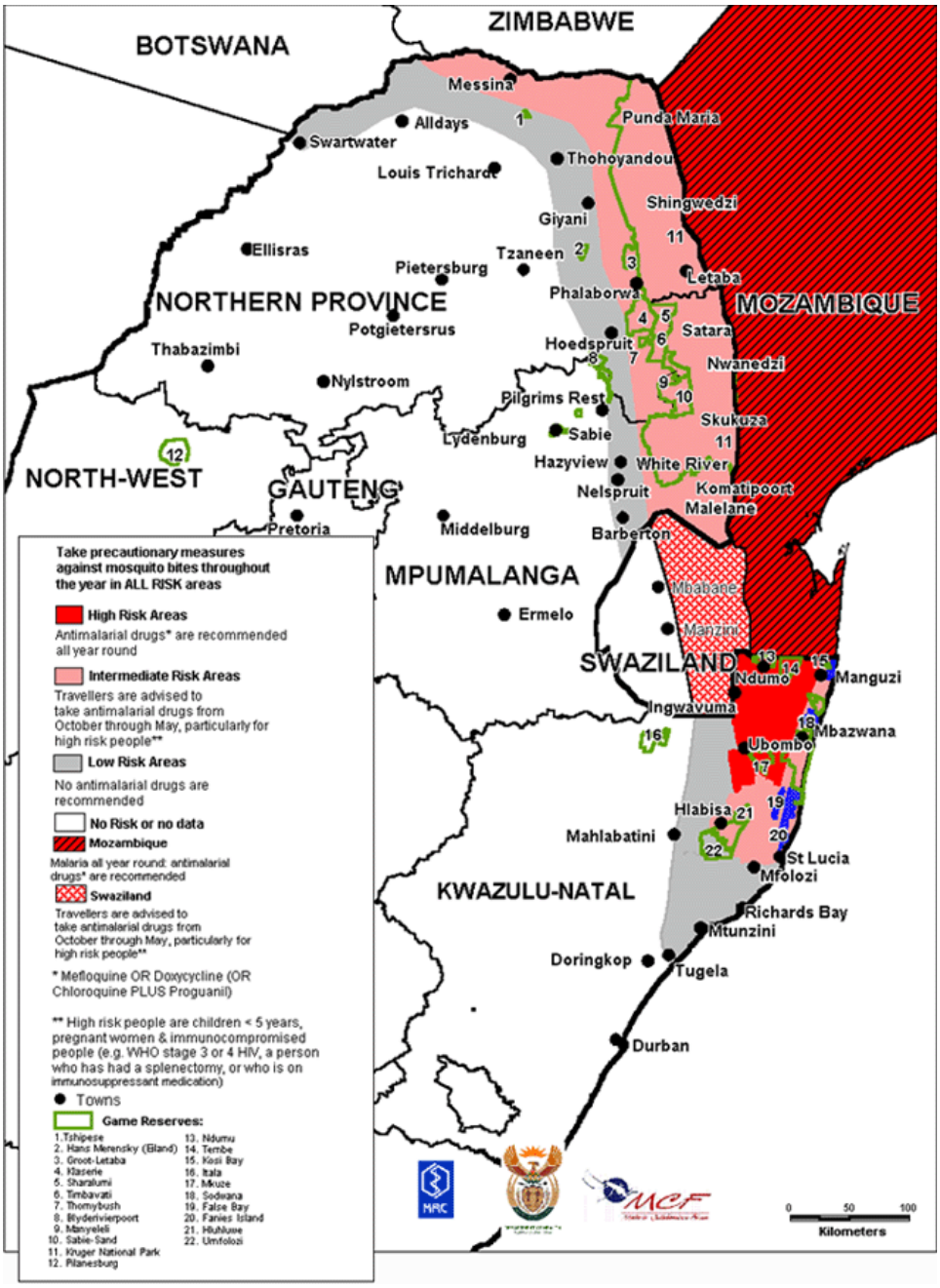


Figure 5.1: Malaria risk areas in South Africa, Swaziland, Lesotho and Mozambique (National Department of Health, 2008a).

5.1.3 Malaria transmission and life cycle

Human malaria is caused by four species of the *Plasmodium* protozoa*; P. falciparum*, *P. malariae*, *P. ovale* and *P. vivax;* with *P. falciparum* accounting for over 90% of human malaria infections and fatalities in sub-Saharan Africa (National Department of Health,

2008a). The life-cycle of the parasite involves two hosts; man and the female *Anopheles* mosquito (Figure 5.2). Sporozoites injected by the infected mosquito during a blood meal (C1) invade hepatocytes and following intracellular growth and multiplication, new parasites are released as merozoites (A4). These merozoites invade erythrocytes (B5), where they can follow one of two developmental pathways. The majority undergo asexual multiplication (B) and are released as daughter merozoites. This cycle lasts for about 48 hours in *P. falciparum* and is associated with the clinical symptoms of the disease. The alternative pathway of development in erythrocytes leads to the formation of male and female gametocytes (d), which are responsible for sexual reproduction and transmission through mosquitoes (C). The latter pathway takes about 14 days in *P. falciparum* (Bell, 2005).

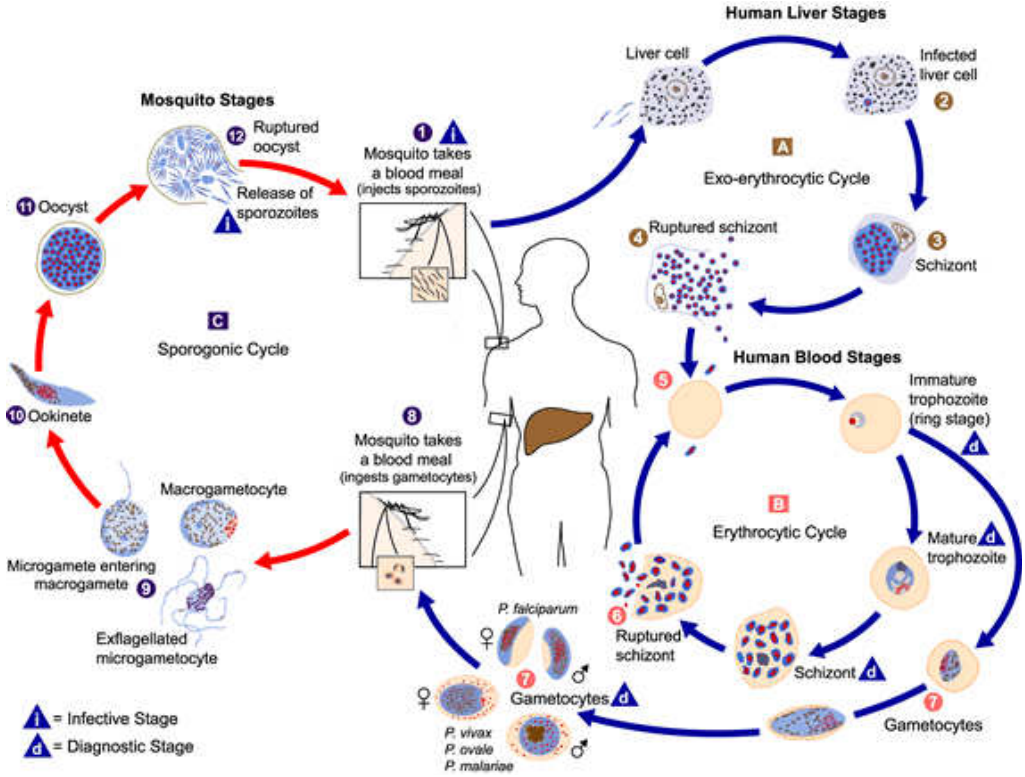


Figure 5.2: The life cycle of the malaria parasite (Centers for Disease Control and Prevention, 2006).

5.1.4 Antimalarial dugs

Antimalarial agents can be classified into five groups according to their biological activity and chemical structure. These are the blood schizontocides, tissue schizontocides for causal

prophylaxis, tissue schizontocides for preventing relapse, gametocytocides and sporontocides (Vangapandu *et al*., 2007).

- *Blood schizontocides*: act on the blood forms of the parasite which result in the termination of the clinical attacks and symptoms of the infection. Drugs included in this group are chloroquine, quinine, mefloquine, halofantrine, pyrimethamine, sulfadoxine, sulfones, tetracyclines and artemisinin and its derivatives (Bell, 2005; Vangapandu *et al*., 2007).
- *The tissue schizontocides for causal prophylaxis:* act on the primary tissue forms of *Plasmodium* within the liver. This stage initiates the erythrocytic stage of development and by blocking it, further development of the infection is prevented. Primaquine and pyrimethamine act against this stage (Bell, 2005; Vangapandu *et al*., 2007).
- *Tissue schizontocides for preventing relapse*: act on hypnozoites (sporozoites that have gone into hibernation) of *P. ovale* and *P. vivax* in the liver which cause relapse of symptoms on reactivation. Primaquine is the only drug used to eradicate this intrahepatic phase (Vangapandu *et al*., 2007; National Department of Health, 2008b).
- *Gametocytocides*: destroy the sexual forms of the parasite in the blood, and thereby interrupting the life cycle by preventing the transmission of the infection from man to the mosquito. Chloroquine and quinine are able to inhibit the gametocyte stage of *P. vivax* and *P. malariae*, but are unable to act against *P. falciparum*. Primaquine and artermisinin-type compounds have gametocytocidal activity against all human malaria parasites (Olliaro, 2001; Vangapandu *et al*., 2007).
- *Sporontocides*: prevent the development of the oocyst in the mosquito and thus stop the transmission from mosquito to human. Primaquine and choloroguanidine are known to have activity against this stage (Vangapandu *et al*., 2007).

5.1.5 Malaria prophylaxis

Malaria prevention includes measures taken against both the mosquito vectors and the malaria parasites. In malaria endemic areas, the use of insecticides, screens on windows, bed-nets, mosquito repellants and fans or air conditioners to disturb mosquito feeding are all measures taken to prevent mosquito bites and possibly contracting malaria. To prevent infection and symptoms, chemoprophylaxis is recommended for high risk groups such as residents in the seasonally endemic areas, pregnant women, young children and travelers to malaria areas. Drugs that are commonly used for chemoprophylaxis in South Africa are mefloquine,

doxycycline and a combination of atovaquone and proguanil with the combination of chloroquine and proguanil kept as a last resort (National Department of Health, 2008a).

- *Mefloquine* is active against all *Plasmodium* parasites, as well as *P. falciparum* resistant to chloroquine and sulfadoxine-pyrimethamine. It is taken at least one week before entering malaria endemic area and continued for four week after leaving malaria area. It is contraindicated during pregnancy and lactation and where fine co-ordination is required, as it may cause spatial disorientation and lack of fine co-ordination (National Department of Health, 2008a). Commonly reported side effects include insomnia, strange dreams, mood changes, nausea, diarrhoea and headache. Severe neuropsychiatric reactions and suicidal ideation are infrequent (Rosenthal, 2006).
- *Doxycycline* is also effective against all four species of human malaria parasites. It is given orally, 100 mg daily and should be started at least one day before entering malaria area and continued for four weeks after leaving malaria area. It is also contraindicated in pregnancy, lactation and during the first eight years in life as it affects bone formation (National Department of Health, 2008a). Commonly reported side effects include gastrointestinal symptoms, and *Candida* infection of the gastrointestinal tract and vagina. Severe skin sensitivity, dizziness headache and blurred vision are rare (Rosenthal, 2006).
- *Atovaquone plus proguanil* is given one day before exposure and continued daily during exposure. Thereafter, it is given for seven days after the last exposure. It is a preferred combination as it appears to have a mild adverse event profile, as well as may significantly increase compliance due to the shortened regimen. Its side effects are nausea, gastrointestinal symptoms, and should be used with caution in patients with renal failure (National Department of Health, 2008a; Rosenthal, 2006)
- *Chloroquine plus proguanil* is given at least one day before entering malaria area. Chloroquine is given weekly, whilst proguanil is given daily. This combination must also be taken for four weeks after leaving the malaria area. It provides some protection against chloroquine-resistant *P. falciparum*. But due to the widespread of chloroquine-resistant strains of *P. falciparum*, this combination is only recommended where the use of mefloquine and doxycycline is contraindicated; otherwise it is no longer in use in South Africa. Serious side effects of this combination are rare, but patients may experience headaches, gastrointestinal effects, skin rashes and mouth ulcers. Periodic eye

examinations are recommended after five years of continuous use (National Department of Health, 2008a; Rosenthal, 2006).

- *Primaquine* is used for the terminal prophylaxis of *P. vivax* and *P. ovale* infections. A daily dose of 26.3 mg is recommended and like any other malaria chemoprophylactic agent, it should be started about a week before entering a malarious area and continued for 14 days after leaving the malaria area. It frequently causes nausea, epigastric pain, abdnominal cramps and headache at high doses, but otherwise it is well tolerated. More serious adverse effects include cardia arrhthmias, leukocytosis, leucopenia ad agranulocytosis, however, these are very rare (Rosenthal, 2006).

In Swaziland, however, chloroquine is still the drug of choice for prophylaxis since no chloroquine resistance has been recorded (National Malaria Control Program, 2004/2005).

5.1.6 Malaria treatment

It is interesting to note that malaria can be successfully treated in 48 hours, however, if diagnosis and treatment are delayed, it can cause fatal complications (Vangapandu *et al*., 2007). The choice of drugs for malaria treatment depends on a number of factors such as the severity of the disease, type of *Plasmodium* parasite, condition of patient such as age, pregnancy, allergies and other medications the patient might be on, as well as resistance pattern of the *Plasmodium* strain.

- Uncomplicated malaria is treated with the artemether–lumefantrine combination or quinine combined with either doxycycline or clindamycin (Rosenthal, 2006).
- For the treatment of *P. falciparum* malaria, quinine, artemether–lumefantrine and intravenous artesunate are used. Due to the development of resistance and adverse side effects sulfadoxine-pyrimethamine, quinine, halofantrine, tetracyclines and clindamycin are no longer in use as a single agent, but tetracyclines and clindamycin are used in combination with quinine (Outtara *et al*., 2006; UNDP, 2005; National Department of Health, 2008b). Amodiaquine in combination with sulfadoxine-pyrimethamine is also used as an alternative in areas where artemisinin containing therapies are unavailable (Rosenthal, 2006).
- When treating non-*P. falciparum*, chloroquine followed by primaquine is recommended (National Department of Health, 2008b).

- Mixed *Plasmodium* infections are treated with quinine or artemether-lumefantrine plus a follow-up course of primaquine (National Department of Health, 2008b).
- Pregnant women with uncomplicated malaria are also treated with quinine followed by clindamycin as it is safe when used in normal therapeutic doses. One of the adverse effects of this drug is hypoglycaemia and patients should be monitored closely. Quinine can also be oxytoxic. There is no known incidence of teratogenesis, but incidence of congenital abnormalities have been reported. In lactating women, artemether–lumefantrine is safely used for uncomplicated malaria. Intravenous quinine is also the drug of choice for infants and young children (National Department of Health, 2008b).
- Severe or complicated infections of *P. falciparum* are treated with intravenous quinidine, artesunate or artemether, the latter two being administered either intravenously or intramuscularly. Cardiac monitoring when administering quinidine is essential as this drug may cause cardiotoxicity (Rosenthal, 2006).

Many of the standard antimalarial drugs have become ineffective because the parasite has developed resistance against them. This has necessitated combination therapy in malaria treatment instead of a single regimen treatment; as well as intensive research into new and unique drugs to ensure the continued control of malaria (Prozesky *et al*., 2001). The search for new drugs can follow three directions; study of *Plasmodium* biochemical pathways, chemical synthesis and phytochemical investigation of medicinal plants (Mbatchi *et al*., 2006).

5.1.7 Herbal remedies in the treatment of malaria

Since time immemorial, plants have been the source of malaria treatment in most parts of the world. The discovery of an alkaloid, quinine from the *Cinchona* L. bark was a breakthrough in malaria treatment and soon, synthetics such as chloroquine and other quinoline antimalarials were made. However, this breakthrough did not last very long as it was soon discovered that the malaria protozoa was able to develop resistance against them, resulting in the development of more dangerous and non-treatable strains of malaria (Taylor and Berridge, 2006). This initiated another search for other alternative compounds to treat malaria. In 1967, a Chinese herb Qinghao (*Artemisia annua*) was discovered to have antimalarial properties; in fact, this had been documented in about 340A.D. for the use in the treatment of fevers in a Chinese "Handbook of prescription for emergency treatment" by Ge Hong (Wright, 2005). Thereafter,

derivatives of artemisinin were synthesized and there was renewed hope for malaria treatment. From past experience, it is known that the protozoa has an exceptional ability to develop tolerance / resistance to treatment (Hastings and Watkins, 2006), the search for new and more efficacious drugs from plant sources must remain an active on-going endeavour.

5.1.8 Herbal remedies used world-wide and in southern Africa

Traditional healers treat malaria according to its "flu-like" symptoms and fever. Most societies in places where malaria is endemic have plants that are traditionally used to treat malaria. Worldwide, plants such as *Terminalia* species are use to treat malaria. In Africa, numerous plants have been identified to possess antimalarial activity, such as *Trichilia, Ficus* and *Ziziphus*. In southern Africa, plants that have been identified and tested for antiplasmodial activity include among others; *Psidium guajava*, *Artemisia afra*, species of *Aloe* and *Ozoroa* (Iwu, 1993; Prozesky *et al*., 2001; van Zyl and Viljoen, 2002; Clarkson *et al*., 2004). With over 24 000 species in southern Africa (Eloff and McGaw, 2006) there is a need to investigate the rich biodiversity for medicinal properties. Plants that have been studied in South Africa for antimalarial activity and have shown activity (IC_{50} < 10 μg/ml) include among others *Ozoroa engleri, Ozoroa insignis, Balanites maughamii, Trichilia emetica, Vernonia colorata, Croton gratissimus, Ziziphus mucronata, Aloe viridiflora, Salvia runcinata* and *Salvia repens* (Prozesky *et al*., 2001; van Zyl and Viljoen, 2002; Clarkson *et al*., 2004; Kamatou *et al*., 2005). In their review of plants tested in South Africa for antimalarial properties, Pillay *et al*. (2008) reported over on 200 species with more than 40% possessing IC_{50} values less than 10μg/ml. Elsewhere in Africa, plants such as *Quassia africana*, *Allanblackia monticola*, *Terminalia macroptera*, *Cajanus cajan*, *Endodesmia calophylloides* and *Sclerocarya birrea* have also shown promising antiplasmodial activity (Mbatchi *et al*., 2006; Gathirwa *et al*., 2008; Duker-Eshum *et al*., 2004; Ngouamegne *et al*., 2008; Sanon *et al*., 2003; Azebaze *et al*., 2007).

5.1.9 Drug combinations in malaria treatment

Many drug-drug interactions have been studied and their effect on malaria parasites (Fivelman *et al*., 1999; Bell, 2005) with synergism being the preferred type of interaction. Quinine has displayed both synergistic and antagonistic interactions in combination with other drugs, for example, a synergistic interaction when combined with artemisinin and arteether (Gupta *et al*.,

2002; Ekong and Warhust, 1990) and antagonism with atovaquone (Canfield *et al*., 1995). The use of monotherapy in malaria treatment is no longer recommended to prevent the development of resistance. Current prophylactic and treatment regimen include the use of atovaquone and proguanil, quinine with either clindamycin or doxycycline, sulfadoxine and pyrimethamine or artemether and lumefantrine, (National Department of Health, 2008a and 2008b). With the possible interactions between drug combinations, the interaction between the concomitant use of standard antimalarials with herbal preparations needs to be investigated. Muregi *et al*. (2007) has investigated the interaction between Kenyan medicinal plants in combination with chloroquine and found both synergistic and antagonistic interaction. Waako *et al*. (2005) also reported an antagonistic interaction between *Aspilla africana* extracts and artemisinin.

With so many plants showing good antimalarial activity and the increased resistance of strains of the malaria parasite to many of the available antimalarial agents, 15 Swazi medicinal plants were selected. The aims of this chapter were to:

- Investigate the possible antimalarial activity of the fifteen Swazi medicinal plants.
- Determine possible pharmacological interactions that may exist between the most active plant extracts and quinine.
- Test for haemolytic activity of the plant extracts to determine a direct inhibitory activity of the plant extracts to the intraerythrocytic parasite.

5.2 Materials and Methods

5.2.1 Protocol

5.2.1.1 Preparation of incomplete and experimental culture media

The incomplete culture medium was prepared as follows: RPMI-1640 (10.4 g; Gibco™), HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethane-sulfonic acid) buffer (5.9 g; Sigma), D-glucose (4.0 g; Merck), hypoxanthine (44 mg; Sigma) and 100 µl gentamicin sulphate (50 mg/ml; Sigma) were dissolved in 1 litre sterile MilliQ™ water. This solution was filter-sterilised using a Sterivex™ 0.22 µm filter unit and stored at +4 ^{o}C until required. To prepare the experimental media, hypoxanthine and gentamicin were excluded from the above recipe.

5.2.1.2 Preparation of sodium bicarbonate and phosphate buffered saline

A 5% (w/v) working solution was prepared by dissolving 50 g $NaHCO_3$ (Saarchem) into 1 litre sterile MilliQ™ water. The solution was sterilized through a 0.22 µm filter and stored at +4 ^oC. To prepare the phosphate buffered saline (PBS, pH 7.4), NaCl (8.0 g), KCl (0.3 g), $Na_2HPO_4.2H_2O$ (0.73 g) and KH_2PO_4 (0.2 g) was dissolved in a litre of sterile MilliQ™ water. The solution was autoclaved for 20 minutes at 120 ^oC and stored at +4 ^oC.

5.2.1.3 Preparation of human plasma

Three bags of human plasma, obtained from the South African Blood Transfusion Services, were thawed, pooled and heat inactivated at 56 ^oC for 2 hours in a water bath. The inactivated plasma was then centrifuged at 3000 revolutions per minute (rpm) for 10 minutes and aliquoted in sterile 50 ml centrifuge tubes. Thereafter it was stored at -20^oC until used.

5.2.1.4 Preparation of complete culture and experimental media

To prepare complete culture media, 10% heat inactivated plasma and 4.2 ml of 5% $NaHCO_3$ were added to either incomplete culture or experimental media (Section 5.2.2.1) to a final volume of 100 ml.

5.2.1.5 Preparation of erythrocyte suspension

Whole blood (South African Blood Transfusion Services) stored in anticoagulant blood tubes containing citrate phosphate dextrose adenosine-1, was centrifuged at 2000 rpm for 5 minutes. The supernatant and buffy coat was aspirated and the pellet re-suspended in PBS (pH 7.4). This was further centrifuged at 2000 rpm for 5 minutes. The supernatant was aspirated and pellet re-suspended in PBS (pH 7.4). This was repeated three times and an equal volume of incomplete experimental media was added to the red blood cell pellet and mixed thoroughly. The erythrocyte suspension was stored at +4 ^oC and used within a week of preparation.

5.2.1.6 *In vitro* culture maintenance of *Plasmodium falciparum*

The chloroquine-sensitive 3D7 strain of *P. falciparum* was continuously maintained on a daily basis in culture according to the method of Trager and Jensen (1976) and van Zyl and Viljoen (2002). A thin blood smear was prepared, fixed with methanol and stained using a rapid haematology staining kit (similar to Giemsa stain) (Laboratory Reagents Service, South

African Institute for Medical Research) and the percentage parasitaemia calculated with the stage of the parasites noted. The culture was maintained at less than 5% parasitaemia. The spent culture media was aspirated off the parasites and replaced with pre-warmed complete culture media. Fresh uninfected human erythrocytes (Section 5.2.1.5) were added to the culture when the parasites were mostly in the trophozoite/schizont stage (Figure 5.3). Parasites were gassed with 5% CO_2, 3% O_2, and 92% N_2 before being incubated at 37 ^oC.

5.2.1.7 Synchronization of the culture

A 5% (w/v) working solution was prepared by dissolving 50 g D-sorbitol (Saarchem) into 1 litre sterile MilliQ™ water. The solution was sterilized through a 0.22 µm filter and stored at +4 ^oC. To keep the culture synchronized, D-sorbitol was added to the predominantly ring-stage cultures (Figure 5.3) every second day. The rationale behind this addition is that ring-stage cultures do not take up D-sorbitol; whilst the trophozoite/schizont cultures osmotically take up D-sorbitol resulting in their lysis. The culture was centrifuged at 1500 rpm for 5 minutes and the supernatant aspirated off. D-sorbitol (20 ml) was added to the parasite, re-suspended and left at room temperature for 20 minutes. Thereafter, the suspension was centrifuged at 1500 rpm for 5 minutes and supernatant aspirated. Complete culture media was added, the culture gassed and incubated at 37 ^oC.

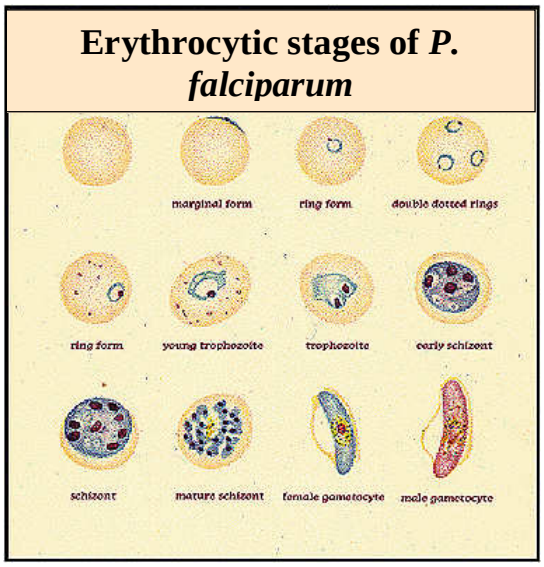


Figure 5.3 Image showing the intraerythrocytic stages of *P. falciparum* (Northwestern University, 2004).

5.2.1.8 Tritiated hypoxanthine incorporation assay

Intra-erythrocytic malaria parasites are incapable of *de novo* purine biosynthesis, which are required for nucleic acid synthesis and energy metabolism. Thus, the purines and especially hypoxanthine are obtained by salvage pathways from the mammalian host. This assay utilises a radiolabelled isotope of hypoxanthine (^{3}H-hypoxanthine), which is incorporated into the parasitic DNA. The amount of ^{3}H-hypoxanthine incorporated is directly proportional to the number of parasitized erythrocytes in culture (Malagon and Castillo, 2000; Daddona *et al*., 1984; King and Melton, 1987; Desjardins *et al*., 1979; van Zyl and Viljoen, 2002). The amount of radioactivity is then determined by β-scintillation counting.

5.2.1.8.1 Preparation of test solutions

The stock plant extract (20 mg/ml) was prepared in dimethyl sulfoxide (DMSO; Saarchem) and stored at +4 ^{o}C until used. From this concentration, a 2 mg/ml solution was prepared in incomplete experimental media. The positive controls, quinine (Fluka) and chloroquine diphosphate (Sigma) were first prepared in DMSO (1 mg/ml) and then incomplete experimental media (30 µg/ml).

5.2.1.8.2 Preparation of experimental plates

Of the concentrations prepared in experimental media (50 µl) was plated in triplicate in the first row of a microtitre plate and serially diluted in a 1:1 ratio in 25 µl experimental media, such that seven concentrations were used to generate the log sigmoid dose response curve. After the first experiment, the initial concentrations were adjusted to ensure an optimal log sigmoid dose response curve. The parasites, predominantly in ring stage, were adjusted to a parasitaemia of 0.5% and 1% haematocrit with complete experimental media. Parasitized red blood cell suspension (200 µl) was added into all, but four wells which contained 200 µl of red blood cells and served as the negative red blood cell control. To account for the 25 µl of extract/positive control added to the test wells, 25 µl experimental media was added to the red blood cell and drug-free parasitized controls. The plate was incubated in a candle jar at 37 ^{o}C for 24 hours in a micro-aerobic and humidified environment (Jensen and Trager, 1977). Thereafter 25 µl 3H-hypoxanthine (5mCi; Amersham Life Science, UK), diluted in hypoxanthine negative incomplete culture media (1:270) was plated into all the wells and further incubated at 37 ^{o}C for 24 hours in the candle jar. The parasite DNA was then harvested using a Titertek® cell harvester onto GFB-filtermats. The filter mats were air-dried

overnight and liquid beta scintillation fluid added before counting the incorporated 3H-hypoxanthine on a Wallac™ beta scintillation counter. The results were expressed as counts per minute (CPM).

The percentage parasite growth was calculated using the equation below:

$$\% \text{ Parasite growth} = \frac{CPM_{extract} - CPM_{mean\ RBC\ control}}{CPM_{mean\ parasite\ control} - CPM_{mean\ RBC\ control}}$$

5.2.1.9 Data analysis

The IC_{50} values (concentration causing 50% parasite death) were determined using the Enzfitter® program (version 1.05) upon plotting log sigmoid dose-response curves. The results are reported as mean ± standard deviation (s.d.) of three separate experiments. ANOVA was used to compare activity between extracts and the controls using Graphpad Prism® (version 4.0). A P value of less than 0.05 ($P<0.05$) was considered significant.

5.2.2 Combination studies

In order to determine the combined effect of the extracts and quinine, the titriated hypoxanthine incorporation assay was used. This is important especially where antagonism exist, because the concurrent use of antimalarial drugs and herbal preparations can result in treatment failure (Bell, 2005). In contrast, a synergistic interaction means that less of the drug needs to be given to achieve the same overall effect as the individual drugs, as well as increase drug efficacy and delay resistance development (Gathirwa *et al*., 2008). Therefore, it is very important to know the kind of relationship that exists as a result of the concomitant use of plant preparations and standard drugs.

The five most active of the extracts were selected and combined with quinine to determine their pharmacological interactive relationship. Different ratios of the plant to quinine were used (Table 5.1). The extract and quinine were prepared at a 20 times higher concentration than shown in the table to account for the 10 times dilution factor in the experiment (Section 5.2.1.8); as well as the 2 times dilution factor generated by mixing the plant extract and quinine in a 1:1 ratio.

Table 5.1: Ratios of plant extracts and quinine used in determining combined antimalarial activity.

Plant	0	0.1	0.5	1	5	10	20	30
Quinine	0.3	0.25	2	1	0.5	0.1	0.01	0

IC_{50} values were determined for each combination as in Section 5.2.1.8.2 and 5.2.1.9. To determine the interaction between the extract and quinine, isobolograms were constructed (Figure 5.4), by plotting the following equation:

$$(X : Y) = \frac{IC_{50} \text{ of quinine in combination}}{IC_{50} \text{ of quinine alone}} : \frac{IC_{50} \text{ of extract in combination}}{IC_{50} \text{ of extract alone}}$$

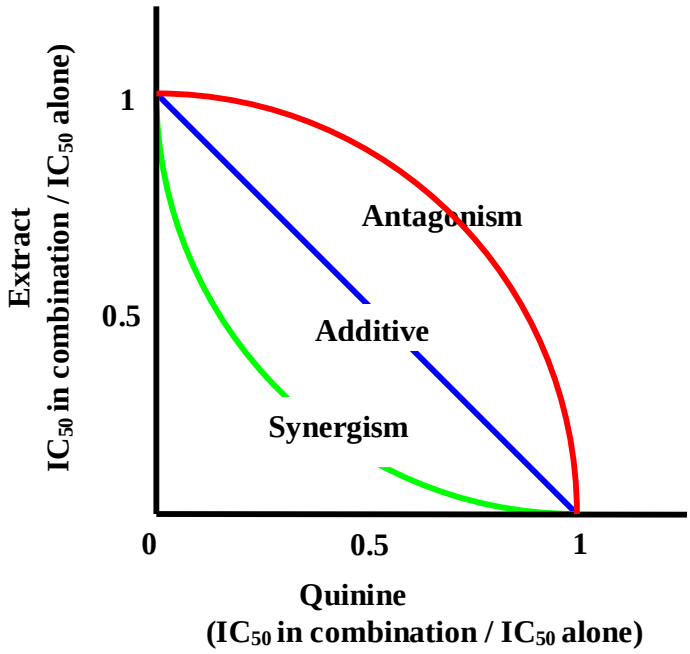


Figure 5.4: Isobologram depicting the possible pharmacological interactions between the plant extract and quinine.

The fractional inhibition concentration (FIC) was also calculated to determine the strength of the interaction using the following equation (Bell, 2005):

$$FIC = \frac{IC_{50} \text{ of extract in combination with quinine}}{IC_{50} \text{ of extract alone}}$$

The sum of the FIC values (ΣFIC) was calculated by adding all the FIC values for all the combination ratios. The proposed definition by Berenbaum (1978) was used to determine the type and strength of the interaction; where a sum of 1 is an additive interaction, <1 a synergistic interaction, and >1 an antagonistic interaction.

5.2.3 Red blood cell haemolysis

A measure of red blood cell integrity is used as a means of determining the cytotoxicity of antiplasmodial agents. Haemolysis is assessed using an *in vitro* assay which shows the effect of increasing concentrations of the extract on the erythrocyte membrane. In this assay, the erythrocytes are exposed to different concentrations of the extract or drug and membrane stability assessed by measuring the amount of liberated haemoglobin using UV spectroscopy (Sharma and Sharma, 2001).

5.2.3.1 Preparation of supplemented PBS and erythrocyte suspension

PBS (100 ml; pH 7.4) (Section 5.2.1.2) was supplemented with HEPES buffer (0.59 g) and D-glucose (0.40 g). The solution was filter-sterilised using a Sterivex™ 0.22 μm filter unit, therafter, plasma (10 ml) was added and the solution stored at +4 ^{o}C until required. A freshly washed erythrocyte suspension (Section 5.2.1.5), was used for this assay. To prepare a 1% (v/v) haematocrit suspension (as used in the 3H-hypoxanthine incorporation assay), packed erythrocytes (1 ml) were added to supplemented PBS (99 ml; pH7.4).

5.2.3.2 Haemolytic assay

A stock of 2 mg/ml and 1mg/ml extract were used in this assay, such that the final concentrations were 200 μg/ml and 100 μg/ml, respectively. Both concentrations of the extract (50 μl) were aliquoted into separate eppendorf tubes before supplemented PBS (50 μl) and the 1% haematocrit erythrocyte suspension (400 μl) were added. This was incubated at 37 ^{o}C for 48 hours to parallel the 48 hour exposure in the tritiated hypoxanthine incorporation assay (Section 5.2.1.8.2). Thereafter, the eppendorf tubes were removed from the incubator, pellet gently disrupted and centrifuged at 2000 rpm for 5 minutes. The supernatant was

removed and plated out (100 µl) in triplicate on a 96-microtitre plate. The plate was allowed to stand at room temperature for 10 minutes before being shaken for 2.5 minutes at 1050 rpm. The absorbance was then read at 540 nm in a Labsystems iEMS Reader MF using the Ascent® software (version 2.4). The following controls were included in the experiment:

- a background control constituting of supplemented PBS only
- a negative control for 0% haemolysis which contained 100 µl supplemented PBS and 400 µl 1% haematocrit erythrocyte suspension and
- a positive control for 100% haemolysis containing 50 µl of 0.2% Triton X-100 (Sigma), 50 µl supplemented PBS (pH 7.4) and 400µl 1% haematocrit erythrocyte suspension.

Percent red blood cell haemolysis was determined using the following equation:

$$\% \text{ Haemolysis} = \frac{(ABS_{extract} - ABS_{BC}) - (ABS_{mean\ NC} - ABC_{BC})}{(ABS_{mean\ PC} - ABS_{BC}) - (ABS_{mean\ NC} - ABS_{BC})}$$

where:
ABS = absorbance;
BC = background control;
NC = negative control;
PC = positive control

For those extracts demonstrating haemolysis at 100 µg/ml (greater than 30%) or 200 µg/ml (greater than 70%), further concentrations were prepared such that a log sigmoid dose response curve could be generated to determine the concentration at which 50% haemolysis occurred.

5.2.3.3 Data analysis

The IC_{50} values (concentration causing 50% cell death) were determined using the Enzfitter® program (version 1.05) upon plotting log sigmoid dose-response curves. The results are reported as mean ± standard deviation (s.d.) of two separate experiments. Within each of the experiments, four replicates were prepared and tested in separate eppendorf tubes. For extracts which had antimalarial IC_{50} values greater than 100 µg/ml, the haemolytic activity at

200 μg/ml was reported. A correlation between haemolytic activity of the extracts and their antimalarial activity was determined using linear regression with a 95% confidence interval. To determine selectivity of some of the extracts for the parasite and the relative toxicity effect of the extracts, the safety index was calculated using the following formula:

$$\text{Safety index} = \frac{\text{Haemolytic activity } (IC_{50})}{\text{Antimalarial activity } (IC_{50})}$$

5.3 Results

5.3.1 Individual antimalarial activity

Antimalarial activity of plant extracts has been widely studied and the parameters for activity suggested by Rasoanaivo *et al.* (1999) are widely accepted. In the present study, the following parameters were used to define activity of an extract; $IC_{50} \leq 5$ μg/ml, highly active, $IC_{50} \leq 5$-10 μg/ml, promising activity, 10-50 μg/ml, moderate, 50-100 μg/ml, low and >100 μg/ml, inactive (Rasoanaivo *et al.*, 1999; Gathirwa *et al.*, 2008). When dealing with isolated compounds, Pink *et al.* (2005) proposed that a compound with $IC_{50} \leq 1$ μg/ml can be considered a 'hit' and that it should be at least tenfold more selective against the parasite than mammalian cell line.

The extracts inhibited parasite growth in a dose-dependent manner (Figure 5.5). The bark of *S. cordatum* was the most active of the extracts, but was still 51.54 fold less active than quinine (Figure 5.5). Table 5.2 illustrates the activity of the extracts against the chloroquine-sensitive 3D7 strain of *P. falciparum*. Overall, 45.45% of the extracts had activity less than 50 μg/ml with 30.30% more than 100 μg/ml. The four extracts that showed promising antimalarial activity with IC_{50} values between 5-10 μg/ml were the leaves of *T. phanerophlebia* (IC_{50} = 7.81 ± 1.82 μg/ml), bark of *S. cordatum* (IC_{50} = 6.70 ± 1.21 μg/ml), bark of *B. salicina* (IC_{50} = 7.41 ± 1.88 μg/ml) and the whole plant extract of *P. meyeri* (IC_{50} = 7.36 ± 0.85 μg/ml). The activity of the latter were found to be significantly different from quinine ($P<0.01$), whilst not significantly different from each other.

The stem/root of *B. setifera*, leaves of *T. emetica* and leaves of *F. glumosa* had moderate activity with IC_{50} values below 20 μg/ml. The latter six extracts did not show any haemolysis

at their respective antimalarial IC_{50} values. The least active of all the extracts, with IC_{50} values greater than 200 µg/ml were the root of *H. acutatum*, the bark of *F. glumosa* and *T. emetica* and the leaves of *H. acutatum* and *G. spatulifolia*. Their percentage inhibition of growth on *P. falciparum* were 76.67 ± 3.56%, 74.84 ± 3.95%, and 71.07 ± 7.78%, 66.14 ± 7.69% and 54.89 ± 4.84%, respectively.

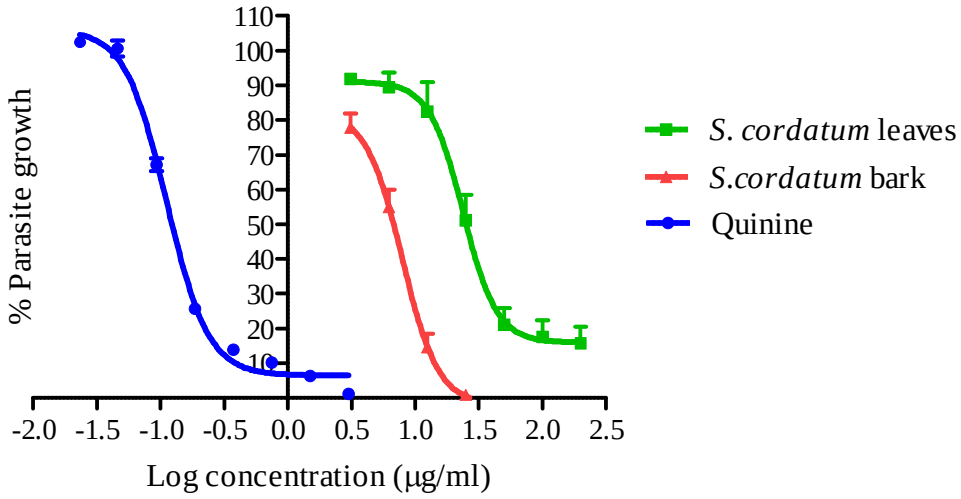


Figure 5.5: Log sigmoid dose-response growth curves for the leaves and bark of *S. cordatum* compared to quinine.

5.3.2 Haemolytic properties

Many of the extracts did not cause more than 20% haemolysis at 100 µg/ml. Only the bark of *O. sphaerocarpa* and *S. cordatum*, as well as the leaves of *F. glumosa* and *B. salicina* caused more than 20% haemolysis at 100 µg/ml (Table 5.2). For those extracts exhibiting haemolysis (Section 5.2.3.2) at 100 or 200 µg/ml, log sigmoid dose response curves were generated and the IC_{50} values determined. These were the bark of *T. emetica* which had an IC_{50} value of 194.38 ± 7.25 µg/ml, leaves of *O. sphaerocarpa* (IC_{50} value of 180.26 ± 9.69 µg/ml), stem/root of *B. setifera* (IC_{50} value of 178.41 ± 33.16 µg/ml), fruit of *D. cinerea* (IC_{50} value of 150.56 ± 20.48 µg/ml) and fruit of *G. spatulifolia* (IC_{50} value of 117.42 ± 10.96 µg/ml). Of these, only the stem/root of *B. setifera* had a safety index greater than 10 (Table 5.3; Table 6.1). There was no correlation between the haemolytic and antimalarial activity of the plant extracts ($r^2 = 0.009$).

Table 5.2: Antimalarial activity and haemolytic properties of the extracts. Values in parenthesis indicate the % parasite growth or % haemolysis at the stated concentration.

Scientific name	Plant part	Antimalarial activity IC_{50} (µg/ml ± s.d.)	Red blood cell haemolysis IC_{50} (µg/ml ± s.d.)
Berkheya setifera	flower	32.3 ± 0.74	>100 (0.47 ± 0.02%)
	stem/root	15.12 ± 1.80	178.41 ± 33.16
	leaves	66.40 ± 4.19	>100 (2.53 ± 0.07%)
Breonadia salicina	leaves	135.79 ± 10.75	>200 (20.31 ± 2.61%)
	bark	7.41 ± 1.88	>100 (9.93 ± 3.73%)
Dichrostachys cinerea	fruit	26.51 ± 1.37	150.56 ± 20.48
	leaves	106.76 ± 9.64	>100 (13.92 ± 1.01%)
Ficus glumosa	fruit	117.54 ± 14.03	>100 (5.91 ± 1.03%)
	leaves	19.40 ± 2.20	>100 (27.43 ± 3.02%)
	bark	>200 (74.84 ± 3.95%)	>100 (19.95 ± 1.33%)
Gardenia spatulifolia	fruit	124.90 ± 9.79	117.42 ± 10.96
	leaves	>200 (54.89 ± 4.84)	>200 (16.01 ± 0.57%)
Guilleminea densa	whole plant	162.32 ±17.38	>200 (5.04 ± 0.28%)
Helichrysum acutatum	leaves	>200 (66.14 ± 7.69%)	>200 (8.15 ± 3.67%)
	roots	>200 (76.67 ± 3.56%)	>200 (1.34 ± 0.08%)
Leonotis intermedia	flower	55.51 ± 7.11	>100 (3.77 ± 0.27%)
	stem	25.98 ± 1.74	>100 (4.05 ± 0.01%)
Ozoroa sphaerocarpa	fruit	40.20 ± 8.54	>100 (1.42 ± 0.02%)
	leaves	54.33 ± 1.24	180.26 ± 9.69
	bark	45.29 ± 2.33	>100 (30.80 ± 5.55%)
Priva meyeri	whole plant	7.36 ± 0.85	>100 (11.11 ± 0.77)
Ricinus communis	fruit	33.61 ± 2.86	>100 (12.18 ± 0.19)
	leaves	23.60 ± 2.28	>100 (13.01 ± 4.11%)
	stem	52.73 ± 0.44	>100 (5.04 ± 0.17%)
Terminalia phanerophlebia	leaves	7.81 ± 1.82	>100 (5.32 ± 1.43%)
	bark	72.62 ± 3.40	>100 (5.25 ± 2.46%)
Trichilia emetica	leaves	18.13 ± 3.55	>100 (6.25 ± 0.68%)
	bark	>200 (71.07 ± 7.78%)	194.38 ± 7.25
Syzygium cordatum	leaves	32.61 ± 2.05	>100 (4.31 ± 0.41%)
	bark	6.70 ± 1.21	>100 (22.83 ± 3.56%)
Ziziphus mucronata	fruit	67.18 ± 7.40	>100 (3.21 ± 0.09%)
	leaves	73.99 ± 1.47	>100 (4.37 ± 0.31%)
	bark	94.84 ± 1.47	>100 (3.44 ± 0.81%)
Quinine		0.13 ± 0.01	>100 (1.56 ± 0.18%)
Chloroquine		0.052 ± 0.003	>100 (1.24 ± 0.02%)

Table 5.3: Antimalarial and haemolytic activity, with safety index of select extracts.

Scientific name	Plant Part	Antimalarial activity IC_{50} (µg/ml)	Haemolytic activity IC_{50} (µg/ml)	Safety index
O. sphaerocarpa	Leaves	54.33	180.26	3.32
B. setifera	stem/root	15.12	178.41	11.80
T. emetica	Bark	>200	194.38	>0.97
D. cinerea	Fruit	26.51	150.56	5.68
G. spatulifolia	Fruit	124.90	117.42	0.94

5.3.3 Combination studies

As seen from the isobolograms (Figure 5.6), there is an additive to antagonistic interaction between the different plant extracts tested in combination with quinine; with a few points showing synergy. The FIC for the individual points was calculated and the results are listed in Appendix D, together with the ΣFIC. The ΣFIC for the combination between *F. glumosa* (leaves) and quinine is 1.69 ± 0.08, depicting an antagonistic interaction. There was also an antagonistic interaction between the bark of *B. salicina* and quinine (ΣFIC = 1.76 ± 0.05), the stem of *L. intermedia* with quinine (ΣFIC = 1.97 ± 0.09) and the leaves of *T. phanerophlebia* with quinine (ΣFIC = 2.05 ± 0.25). The stem/root of *B. setifera* with quinine was the most antagonistic of all with ΣFIC of 5.52 ± 0.34.

5.4 Discussion

Our results have shown potential antimalarial activity in some of the plants tested (Table 5.2) indicating that traditionally used medicinal plants from southern Africa do have potential as possible sources of antimalarial compounds. The bark of *S. cordatum* was the most promising of the extracts tested (Table 5.2). In a previous study by Clarkson *et al.* (2004), the dichloromethane/methanol (1:1) extract of the twigs of *S. cordatum* were shown to possess antimalarial activity with an IC_{50} value of 14.7 µg/ml, when tested on the chloroquine-sensitive D10 strain using the parasite lactate dehydrogenase (pLDH) assay. This is the same extractant used in this study, the difference being that the bark material rather than the twigs was used, which gave a lower IC_{50} value of 6.70 ± 1.21 µg/ml (Table 5.2). This difference could be due to the bark being more potent than the twigs because the bark, being an older part of the plant, may have accumulated many of the active metabolites than the newer twigs. The bark is preferentially used by the traditional healers (PP Ndlovu, personal communication).

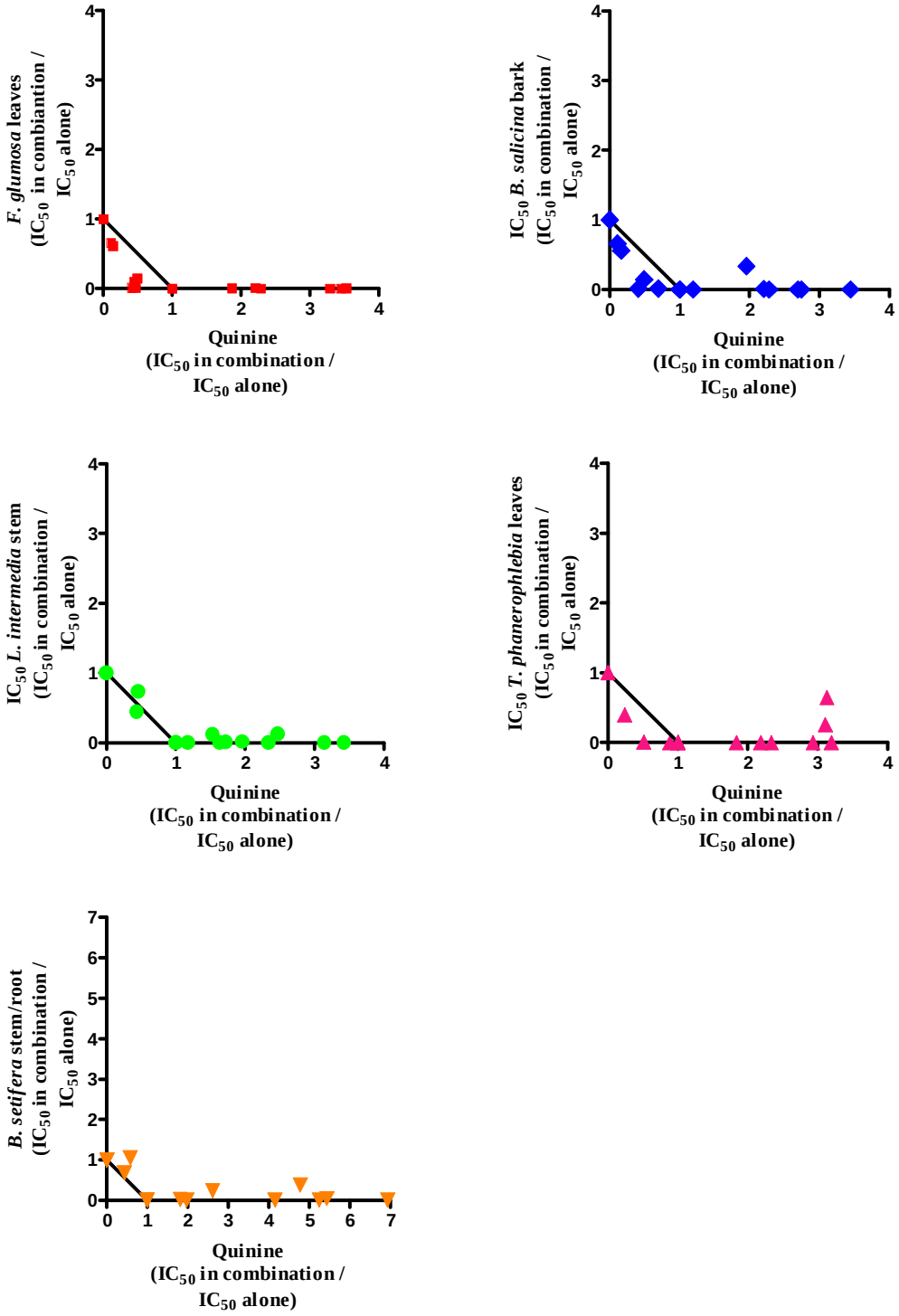


Figure 5.6: Isobolograms depicting the pharmacological interactions between *F. glumosa*, *B. salicina*, *L. intermedia*, *T. phanerophlebia* or *S. setifera* in combination with quinine.

Secondly, the differences in IC_{50} values could have been a result of experimental variation between the assays, the parasite strain and the percent parasitaemia and haematocrit used (van Zyl, 2007). *Syzygium claviflorum* is known to contain the triterpene betulinic acid; which has anti-HIV properties (Crump, 2006), as well as antiplasmodial activity with IC_{50} values ranging between 19.4-19.6 µg/ml on the chloroquine-sensitive 3D7 strain and the chloroquine and pyrimethamine-resistant K1 strain, respectively (Steele *at al.*, 1999; Duker-Eshun *et al*., 2004).

As reported in Chapter 3, such compounds as friedelin, epi-friedelin, gallic acid, ellagic acid and delphinidin have been isolated from *S. cordatum*. Of these, the antimalarial activity of friedelin and ellagic acid has been tested. Friedelin was found to possess antimalarial activity with an IC_{50} value of 7.2 ± 0.5 µg/ml on the chloroquine-resistant W2 strain (Ngouamegne *et al*., 2008), whilst Azebaze *et al*., (2007) did not find any activity (IC_{50} > 200 µM) using both the chloroquine-sensitive F32 and chloroquine-resistant FcM29 strains. Ellagic acid has been reported to possess antimalarial properties with IC_{50} value of 0.14 ±0.05 µg/ml on the chloroquine-resistant FCR-3 strain (RL van Zyl, personal communication). The activity observed in this plant may therefore be due to synergistic interactions between the different constituents, as the leaves also contained phenolics and flavonoids (Table 7.2).

This is the first report on the promising activity of the leaves of *T. phanerophlebia* (IC_{50} = 7.81 ± 1.82 µg/ml). A study by Mustofa *et al.* (2000) found antimalarial activity in the stem and leaf ethanolic extracts of *T. glaucescens* with (IC_{50} values ranging from 4.83 ± 0.006 µg/ml to 2.41 ± 0.001 µg/ml, using the FcM29-Cameroon chloroquine-resistant, FcBI-Coloumbia chloroquine-resistant and Nigerian chloroquine-sensitive strains. A study on the same species revealed activity with IC_{50} values as low as 1.80 µg/ml on the alkaloid extract on the FcB1 strain (Okpekon *et al*., 2004). Sanon *et al*. (2003) also found activity of the root bark aqueous extract (IC_{50} = 1 µg/ml) of *T. macroptera* on the chloroquine-resistant W2 strain, using a flow cytometric method. The stem bark and stem wood of *T. spinosa* were also found to possess antimalarial activity when tested on the ENT36 chloroquine-resistant and K67 chloroquine-sensitive strains (Omulokoli *et al*., 1997). Their results are in agreement with those reported here (Table 5.2), validating the antiplasmodial activity of the *Terminalia* species and their use by traditional healers in southern Africa and the Ivory Coast.

Trichilia emetica has also been widely studied for antimalarial activity and the *Trichilia* genus has been shown to have general antiplasmodial properties (MacKinnon *et al*., 1997). The result found in this study (IC_{50} = 18.13 ± 3.55 μg/ml) is very similar to that reported by El Tahir *et al*. (1999) who tested the activity of a leaf methanolic extract on the same strain of *P. falciparum* used in this study (chloroquine-sensitive 3D7 strain; IC_{50} = 17.5 μg/ml). Clarkson *et al*. (2004) reported antiplasmodial activity of a dichloromethane/methanol (1:1) extract of the leaves/twigs of *T. emetica* with an IC_{50} value of 3.5 μg/ml on the chloroquine-sensitive D10 strain using the pLDH assay. While, Prozesky *et al*. (2001) reported activity of a dichloromethane extract of the stem bark on the chloroquine-resistant PfUP1 strain using the flow cytometric method (IC_{50} = 3.29 μg/ml). In this study, the bark dichloromethane/methanol extract did not show any activity, only inhibiting 28.93% of parasite growth at 200 μg/ml (Table 5.2). Whilst El Tahir *et al.* (1999) reported high activity (IC_{50} = 8.5 μg/ml) of the stem bark methanolic extract against the same 3D7 strain, but a similar low activity (IC_{50} = 200 μg/ml) was also reported against the chloroquine-resistant/pyrimethamine-sensitive Dd2 strain. In addition, Bah *et al*., (2007) reported activity of the leaf dichloromethane extract (IC_{50} = 11.90 μg/ml) using the 3D7 strain of *P. falciparum*. The results in this study confirm the antiplasmodial activity of the *Trichilia* genus, however the different strains used and methodology (% parasitaemia) could have influenced the higher IC_{50} value obtained on the 3D7 strains in comparison to the chloroquine-sensitive D10 and chloroquine-resistant PfUP1 strains (Table 5.2). Other factors that could have led to the loss of the active components or contributed to the differences in activity include geographical location, seasonal variation, and age of plants or extraction procedures (Pillay *et al*., 2008). The antiplasmodial activity of this plant has been attributed to the general activity of the family Meliaceae which is reported to contain limnoids such as gedunin and its derivatives which possess antimalarial activity (Schwikkard and van Heerden, 2002).

Following a literature survey, this appears to be the first report on the antimalarial activity of *Ficus glumosa*. *Ficus gnaphalocarp* and *F. platphylla* are reportedly used by the Ghanaian people for the treatment of malaria symptoms (Asase *et al*., 2005). The *Ficus capensis* leaf ethanolic extract was found to have some *in vitro* antiplasmodial activity when tested against the chloroquine-resistant FcB1/Columbia strain using the hypoxanthine incorporation assay with an IC_{50} value of 45.3 ± 5.1 μg/ml (Zirihi *et al*., 2005). While results for the *F. glumosa*

leaf extract showed more potent activity on the 3D7 strain of *P. falciparum* with an IC_{50} value of 19.40 ± 2.20 μg/ml; whilst the bark extract only inhibited 25.16% of parasite growth at 200 μg/ml (Table 5.2). The antimalarial properties may be due to the presence of pentacyclic triterpenes in the plant as some activity has been reported from triterpenes from other species (Suksamrarn *et al*., 2003; Ngouamegne *et al*., 2008).

Of the remaining plants, the bark of *B. salicina*, *P. meyeri* (whole plant) and the stem/root of *B. setifera* showed promising to moderate antiplasmodial activity with IC_{50} values of 7.41 ± 1.88 μg/ml, 7.36 ± 0.85 μg/ml and 15.12 ± 1.80 μg/ml, respectively (Table 5.2). However, a literature search revealed that these plants have not previously been investigated for antiplasmodial activity. These plants should be investigated further to confirm the observed activity on other strains with different drug-resistant properties. The latter could be very promising sources of antimalarial compounds since they did not cause red blood cell lysis at the concentration required to inhibit 50% parasite growth. However, *P. meyeri* was very toxic to human kidney epithelial cells with IC_{50} value of 14.63 ± 1.62 μg/ml, with a safety index of 1.99 (Table 6.1). This warrants further research and isolation of the active antimalarial compound to determine whether or not it is the same as that responsible for mammalian cell toxicity. The extracts of *B. salicina* and *B. setifera* were not toxic to human kidney epithelial cells and may be a valuable source of an antimalarial compound as nothing has reportedly been isolated from these plants.

In a study conducted by Clarkson *et al*. (2004), three *Leonotis* species were investigated for antimalarial activity, namely *L. leonurus, L. ocymifolia* and *L. nepetifolia,* with IC_{50} values ranging between 2 and 30 μg/ml on the chloroquine-sensitive D10 strain. Of note was the dichloromethane: methanol (1:1) extract of the leaves or twigs of *L. leonurus*, which had a promising activity (IC_{50} = 5.4 μg/ml). Results in this study showed the activity of the stem extract of *L. intermedia* to be similar to *L. leonurus*, with an IC_{50} value of 25.98 ± 1.74 μg/ml. However, nothing has been reported on the antimalarial activity of its significant constituent marrubiin (Ah Kee *et al*., 2008).

In this study, the extract of the root and leaves of *Helichrysum acutatum* is reported as being inactive, inhibiting approximately 30% parasite growth at 200μg/ml (Table 5.2). A similar inactivity was reported by van Vuuren *et al*. (2006) who reported low activity (IC_{50} = 60.76 ±

2.83 μg/ml) for the acetone extract of *H. cymosum* on the chloroquine-resistant FCR-3 strain. Where the compound, helihumulone was isolated and found to have a higher antiplasmodial activity (IC_{50} = 14.89 ± 0.88 μg/ml) than the crude plant extract. In contrast, Clarkson *et al.* (2004) reported high activity of the whole plant dichloromethane/methanol extract of *H. nudifolium* (IC_{50} = 6.8 μg/ml) on the chloroquine-sensitive D10 strain. The plant sap of *H. panduratum* is reported to be used for the treatment of malaria in children (Lourens *et al*., 2008); but no *in vitro* activity has been reported.

The antimalarial activity of the leaf extract of *Ricinus communis* reported in this study (IC_{50} = 23.60 ± 2.28 μg/ml) was supported by that of Clarkson *et al.* (2004) who reported on the activity of a leaf dichloromethane/methanol extract (IC_{50} = 27.5 μg/ml). However, there is a seven fold difference between the reported activities of the respective stem extracts; where Clarkson *et al*. (2004) reported a high activity (IC_{50} = 8.00 μg/ml), in contrast to the poor activity (IC_{50} = 52.73 ± 7.11 μg/ml) observed in this study. No antiplasmodial activity is recorded for the constituents of *R. communis* like ricin and ricinine.

In stark contrast to the potent antimicrobial activity (Table 4.1), *Ozoroa sphaerocarpa* possessed poor antimalarial activity (Table 5.2). A previous study on *O. engleri*, a dichloromethane stem bark extract was shown to possess very good activity (IC_{50} = 1.70 μg/ml) (Prozesky *et al*., 2001). *Ozoroa insignis* is also used to treat malaria symptoms by the Ghanaian people (Asase *et al*., 2005), and has been documented to possess antimalarial activity (Gessler *et al*., 1994; Pillay *et al*., 2008).

The bark dichloromethane extract of *Z. mucronata* has been reported to have high antimalarial activity (IC_{50} < 5 μg/ml) (Prozesky *et al*., 2001), however this was not observed in this study where the concentration required to inhibit 50% growth was in the range of 67.18 ± 7.40 to 94.84 ± 1.47 μg/ml. This observation is in agreement with the lack of antimalarial activity of the leaf dichloromethane: methanol extract of *Z. mucronata* (IC_{50} > 100 μg/ml) reported by Clarkson *et al*. (2004). Suksamrarn *et al*. (2005) has reported actitvity of two cyclopeptide alkaloids, ziziphine N and ziziphine Q from Z. *oenoplia* var. *brunoniana*, with IC_{50} values of 3.92 and 3.5 μg/ml, respectively; indicating that species and locality are important variables influencing the antimalarial activity.

The fruit and leaves of *G. spatulifolia* did not possess any antimalarial activity with the IC_{50} value of the fruit being 124.90 ± 9.79 μg/ml; whilst the leaves only resulted in 49.11% parasite death at 200 μg/ml (Table 5.2). This is in agreement with a study by Suksamrarn *et al*. (2003) who reported no activity for the methanol extract of the twigs of *G. saxatilis*. However, in the same study, the chloroform and hexane extracts had antiplasmodial activity which resulted in the isolation of 10 triterpenes, with four having antiplasmodial activity. These were messagenic acid A and B with IC_{50} values of 1.50 and 3.80 μg/ml, respectively, uncarinic acid E and 27-p-(E)-coumaroyloxyursolic acid, both having IC_{50} values of 2.90 μg/ml. *Gardenia tenifolia* is reported to be used by the Ghanaian people for the treatment of malaria (Asase *et al*., 2005).

Dichrostachys cinerea showed variable activity among its parts, with the fruit possessing moderate antimalarial activity (IC_{50} = 26.51 ± 1.37 μg/ml); whilst the leaves did not show any significant activity (IC_{50} = 106.76 ± 9.64 μg/ml) (Table 5.2). When correlating these results to the toxicity results (Table 6.1), it can be seen that the fruit was also toxic to these mammalian cells and it is possible that the activity reported here is due to the general cytotoxicity of the fruit since the fruit pericarp and seeds were not separated during extraction. Further cytotoxicity studies against other human cell lines are required. Both the fruit and the leaves did not show any red blood cell toxicity (Table 5.2) which excludes haemolysis of the red blood cell membrane as the antimalarial mode of action. No antimalarial activity has previously been reported for *D. cinerea*. However, the stem bark acetone extract of another plant from the same family, *Acacia xanthoploea* Benth showed promising activity when determined by a flow cytometric assay on the chloroquine-resistant PFUP1 strain (IC_{50} = 10.10 μg/ml) (Prozesky *et al*., 2001).

Guilleminea densa (whole plant) did not possess any antimalarial activity (Table 5.2). There is no previous research to be found on this plant. One genus from the Amaranthaceae family has been reported to possess some antimalarial properties in South Africa, namely *Achyranthes aspera* L. which was tested on the chloroquine-sensitive D10 strain using the pLDH assay. An IC_{50} value of 9.90 μg/ml was obtained for the dichloromethane: methanol (1:1) extract of the whole plant (Clarkson *et al*., 2004).

Most of the extracts with the exception of the leaves of *F. glumosa* and *B. salicina* and the bark of *O. sphaerocarpa* and *S. cordatum* did not possess red blood cell toxicity (Table 5.2).

This implies that the mechanism of action against *P. falciparum* is not related to red blood cell lysis, but rather to a direct inhibitory effect on the intra-erythrocytic parasite (Sharma and Sharma, 2001).

All the plant/quinine combinations that were tested in this study resulted in antagonistic interactions with ΣFIC values greater than 1 (Section 5.3.3; Figure 5.6). However, upon examining the individual FIC values of the different combinations, it was noted that the overall ΣFIC does not necessarily reflect what is happening within the different ratios of plant to quinine combination. In some cases for example, the leaves of *F. glumosa*, give antagonistic interaction at the high quinine: extract concentrations. Upon reducing the quinine and increasing the extract concentration, a synergistic interaction is obtained. The same was observed for the bark of *B. salicina*. An additive interaction was also observed at the lowest concentration of quinine to the stem of *L. intermedia* (Appendix D).

The mechanism resulting in these interactions cannot be known at this stage since the mechanism of action of extract and its many active components are unknown. It is known that quinine, like the other quinoline drugs, acts on the intra-erythrocytic stage of the parasite, where its mechanism of action involves the inhibition or termination of haemozoin formation. After the parasite has entered the red blood cell, haemoglobin is ingested and sequestered in the food vacuole resulting in the release of haem (Fe(II)PPIX). In the presence of oxygen, the toxic haem is oxidized to Fe(III)PPIX which are polymerized to form haemozoin. In the presence of the quinolines, crystallization of the haem is prevented by the drug forming drug-haem complexes. These latter complexes then damage the parasite membranes and enzymes via lipid peroxidation (Olliaro, 2001; Becker *et al*., 2004; Vangapandu *et al*., 2007; Egan, 2008).

The extract/quinine combinations predominantly show antagonism at most concentrations tested. This antagonism could be due to the extracts selectively entering and changing the pH of the food vacuole, resulting in the inhibition of the uptake of quinine. Quinine is a weak base; any change in pH affects the ionization of the compound so that the concentration accumulating in the food vacuole is decreased (Olliaro, 2001). It is also possible that some of the extracts like *T. phanerophlebia* leaves and *F. glumosa* bark, which possesses iron chelating properties (Table 7.1), antagonize quinine by preventing the formation of the toxic drug-haem complex (Mabeza *et al*., 1999). It is also possible that the extract and drug compete for the

same uptake mechanism or that there is formation of extract-drug complexes which prevent cellular drug uptake (Waako *et al*., 2005), which would explain why there is synergism when there are low concentrations of the quinine to extract (Figure 5.6).

Similar observations of both antagonistic and synergistic interactions have been reported between standard antimalarials and plant extracts. In a study on the antimalarial activity of plants used in Kenya to treat malaria symptoms, varied interaction between the plant extract and chloroquine were reported (Muregi *et al*., 2007). Of note is the antagonistic interaction for the leaf and root-bark of *Ficus sur* on chloroquine-tolerant NK65 strain of *P. berghei*, using the *in vivo* model. This is consistent with the antagonistic interaction of *F. glumosa* and quinine observed in this study (Figure 5.6). Waako *et al*. (2005) also reported antagonism between the shoot extract of *Aspilia africana* and artemisinin using the *in vitro* model on the chloroquine-sensitive D10 strain and chloroquine and sulphonamide-resistant K1 strain.

The preferred interaction in clinical practice is that of synergy. Synergistic combinations in malaria treatment is not a new phenomenon; current regimes use different combinations of drugs to increase the efficacy of the treatment, for example, the use of sulfadoxine in combination with pyrimethamine for the treatment of uncomplicated malaria in areas with chloroquine resistance. However, this combination is no longer recommended in South Africa and artemether with lumefantrine has replaced it. Chloroquine in combination with proguanil was previously used for prophylaxis; however, with the high levels of chloroquine resistance, it is no longer in use. Atavaquone and proguanil are currently in use for causal prophylaxis (National Department of Health, 2008a, 2008b).

Synergistic combination therapy also has the benefit of using low doses of the single agent and thereby reducing its adverse effects (Williamson, 2001) as well as reducing the possibility of resistance development by a parasite to a single agent (Berenbaum, 1989). Synergism between two drugs might result from binding to the same target protein, binding to a transporter that enhances the uptake of the second drug, formation of a complex between the two drugs that increases their potency or one might stimulate the conversion of one to a more potent or active form (Bell, 2005), or further still, through sequential inhibition of enzymes, for example pyrimethamine and sulphadoxine, which act by inhibiting dihydrofolate reductase and dihydropteroate synthase, respectively in the folate synthesis pathway (Olliaro, 2001).

Bell (2005) defines synergism as an effect of drugs in combination that is higher than would be expected from the individual activities. It is very important that we know the kind of interaction to be expected when combining standard antimalarial drugs with traditional medicines. This is especially relevant in the rural areas where it is possible for an individual to have consulted traditional healers for the treatment of malaria symptoms before resorting to western medicine. In this case, the individual might have possibly taken some traditional medicine which may result in synergistic, additive or antagonistic interactions when taken with standard antimalarial drugs. The latter interaction is the most worrying, as this could result in therapeutic failure and possible fatalities if adequate regimens are not administered.

It can be concluded that some of the extracts, such as the bark of *S. cordatum*, leaves of *T. phanerophlebia*, bark of *B. salicina* and whole plant extract of *P. meyeri*, do possess antimalarial properties and there is need for further investigation. Some of the extracts, for example *T. emetica* and *R. communis*, are documented as antimalarial agents in traditional medicine, but did not show any antiplasmodial properties in this investigation. These extracts are probably not acting directly on the parasite, but are used to reduce malaria symptoms such as fever, or they may act as pro-drugs and are metabolized into active drugs such as in the case of the inactive proguanil, which is metabolized to the active form cycloproguanil or artemisinin to dihydroartemisinin (Rosenthal, 2006). It would also be worthwhile to investigate the effect of the extracts on the sexual stages of the parasite, because though they may not be active on the asexual stages, they may have an effect on the gametocyte stages such as the artemisinin-type drugs (Olliaro, 2001). The study has further confirmed the importance of ethnobotany in antimalarial drug research.

CHAPER 6: MAMMALIAN CELL TOXICITY

6.1 Introduction

6.1.1 Plant toxicity

The World Health Organization has set out, among its criteria, that in order for herbs to be used as medicine, they should be shown to be non-toxic (WHO, 1978). Plants commonly used in traditional medicine are assumed to be safe and free from side effects because they have been in use for the treatment of diseases for a long time. However, in addition to the historical information about their use, a formal toxicological evaluation of the plant should be carried out before they are accepted as safe medicine. This is because recent scientific research has shown that many plants that are used as food or medicine are potentially toxic, mutagenic and carcinogenic (Fennell *et al.*, 2004; Buenz *et al*., 2007; Mukinda and Syce, 2007; Sowemimo *et al*., 2007).

Toxicology is mainly concerned with adverse or deleterious effects in living organisms resulting from an exposure to a toxic substance, either a drug or chemical substance. Exposure can either be by inhalation, ingestion or by transdermal means. Toxic reactions differ depending on the length of time the individual is exposed to the toxic substance and can either be acute (single exposure or occurring over one to two days) or chronic (prolonged exposure) (Klaassen, 2001; Plaa, 2007). The symptoms associated with exposure to toxic substances, especially plant products, vary from mild gastrointestinal symptoms and allergic reactions to renal and hepatic toxicity, cardiovascular, neurological complications, haematological, carcinogenic effects and death (Yahnbaş *et al*., 2006).

Due to the complexity of herbal preparations, it becomes difficult to determine the agents responsible for the adverse reactions seen in exposed patients. However, it is well known that herbal preparations containing amongst others aristolochic acid, aconitine, podophylline, achiotoxin, pyrrolizidine alkaloids or atractyloside can cause toxicity. In addition, microbial and heavy metal contamination of these plants may be responsible for their observed toxicity. In some cases, adulterations of herbal medicines with pharmaceuticals can result in the adverse effects observed after their ingestion (Coulombe *et al*., 1999; Snyman *et al*., 2005; Yahnbaş *et al*., 2006).

6.1.1.1 Major organs affected by intoxication with herbal preparations

The dose-response principle is essential when estimating the severity of a herbal intoxication. In fact, this is true with all chemical substances, even pharmaceutical drugs. There is no safe or toxic chemical; safety depends on the dose taken, as well as the risk associated with the use of the chemical substance (Klaassen, 2001). As an example, water and oxygen, which are quite essential for life can be toxic in excessive amounts (Bandaranayake, 2006). Also, paracetamol (acetaminophen), commonly used for mild to moderate pain and as an antipyrectic, is highly toxic to both the liver and kidney in high doses (Furst and Ulrich, 2007).

Many plant toxins are central nervous system depressants and death results from airway obstruction due to a flaccid tongue, aspiration of gastric contents into the tracheobronchial tree or respiratory arrest. Examples of these are podophyllotoxins from the rhizome of *Podophyllum hexandrum*, which results in sensory neuropathy after ingestion (Deng, 2002). Cardiovascular toxicity is also seen in plant or herbal preparation poisoning with symptoms such as hypotension, hypovolemia, peripheral vascular collapse or cardiac arrhythmias being observed. Ingestion of aconitine obtained from *Aconitum carmichaeli* and *Aconitum kusnezoffii* results in cardiotoxicity evidenced by hypotension and ventricular ectopics (Chan *et al*., 1993; Yoshioka *et al*., 1996; Deng, 2002). Cellular hypoxia is evidenced by tachycardia, hypotension, severe lactic acidosis and signs of ischaemia on the electrocardiogram. Renal and hepatic toxicity is seen after ingestion of aristolochic acid-containing preparations, as well as those containing atractyloside and its metabolites. Such compounds are found in plants such as *Symphytum* spp, *Dryopteris* spp, *Callilepis laureola* and *Lycopodium serratum* (Popat *et al*., 2001; Deng, 2002).

The more severe and chronic result of intoxication is DNA damage (genotoxicity). This form of toxicity will not present with acute signs such as allergy reactions, gastrointestinal problems, but will take time to manifest. Plants containing pyrrolizidine alkaloids, such as *Senecio* spp, *Symphytum* spp, *Crotalaria* spp, *Tussilago* spp and *Heliotropium* spp, have been shown to be genotoxic. Pyrrolizidine has been shown to form crosslinks with DNA and proteins in humans (Coulombe *et al*., 1999).

6.1.1.2 Prevalence of traditional medicine poisoning in South Africa

Currently, the prescription and use of traditional medicine in South Africa is not regulated. This results in the risk of misadministration and microbial contamination of these plants resulting in their toxicity. Adulteration of herbal remedies also poses a threat for their safe use. Adulteration of Chinese herbal preparations with heavy metals, pharmaceutical drugs and other toxic plants has had adverse effects on the patients resulting in hepatic, renal and neurotoxic effects (Fennel *et al*., 2004; Snyman *et al*., 2005).

In South Africa, data on traditional medicine poisoning is not reflective of the real scenario. This is because some of the cases happen in rural areas and the people die without even going to hospital. Even with those cases that do reach hospitals, there is lack of analytical equipment to determine the active compound responsible for the poisoning. In addition, people who use traditional medicine may not divulge the information about the remedy they took to the doctor and the poison-linked symptoms may be associated with the illness the remedy was initially taken for (Stewart *et al*., 1999; Popat *et al*., 2001).

Estimates are that traditional medicines are responsible for about 51.7% of all acute poisoning deaths (Foukaridis *et al*., 1994). In another study by Stewart *et al*. (1999), 62% of the poisoning cases studied at the forensic science service of the Gauteng province were due to poisoning by a traditional remedy. In addition to this, the many potentially mutagenic South African medicinal plants can cause long term damage when administered. This calls for cautious use of traditional medicines and the need for toxicological assessment before they are prescribed as medicines (Fennell *et al*., 2004).

Thus, the aim of this Chapter was to determine the toxicity of the plants used in Swazi traditional medicine using an *in vitro* model; and using the reported antimalarial activity (Table 5.2) to calculate the safety index of the plants.

6.2 Materials and Methods

6.2.1 Tetrazolium based cellular viability assay

The 3-[4,5-dimethylthiazol-2yl]-2,5diphenyltetrazolium bromide (MTT) assay is based on the principle that metabolically active cells react with the MTT reagent (pale yellow) to produce

dark blue formazan crystals. The colour change is then read in a multiwell plate reader at a test wavelength of 540 nm and a reference wavelength of 690 nm. The amount of formazan produced is directly proportional to the number of live cells (Mosmann, 1983).

6.2.2 Protocol

6.2.2.1 Media preparation

Experimental media was prepared using 9.38 g Ham F10 media (Highveld Biological) supplemented with 1.18 g $NaHCO_3$ (Saarchem) into 1 litre MilliQ® water. Culture media was prepared exactly the same way, differing only in the addition of 50 mg/ml gentamicin (Sigma). Thereafter, it was filter sterilized through a 0.22 µm filter (Millex™). To optimize cell growth, 5% (v/v) heat inactivated fetal calf serum (heat inactivated at 56 ^{o}C for 1 hour) was added before use.

6.2.2.2 Preparation of extracts / positive control

A stock concentration of 20 mg/ml for the extract, 30 mg/ml for quinine (Fluka) and chloroquine diphosphate (Sigma) and 10 mg/ml for amphotericin B and ciprofloxacin (CA Milsch) was prepared in DMSO (Saarchem) and aliquoted into sterile eppendorf tubes, stored at -20 ^{o}C until required. To prepare the first working concentration of the extract, a 2 mg/ml concentration and subsequent dilutions were prepared taking into account the 10 times dilution factor in the experiment, to give final concentrations ranging from 200 to 1 µg/ml. These dilutions were prepared in experimental media just before being added to the cells for the assay.

6.2.2.3 Culture maintenance

The transformed human kidney epithelial (Graham) cells were continuously maintained in culture at 37 ^{o}C in 5% CO_2 (Ethics clearance waiver number W-CJ-090424-10; Appendix C1 and Biosafety Clearance number IB090503). The culture media was replaced twice weekly. The cells were trypsinized weekly after reaching confluency with 0.25% Trypsin-0.1% Versene-ethylenediaminetetraacetic acid disodium (versene-EDTA; Highveld Biological), that is, after aspirating off spent culture media, trypsin / versene-EDTA (4 ml) was added to the culture flask and incubated at 37 ^{o}C for 5 minutes. This was done to lift the cells from the bottom of the flask. Thereafter, culture media (6 ml) was added to neutralize the trypsin and

the cells centrifuged at 1500 rpm for 5 minutes (Sorvall® T6000D). The media was aspirated off and the cells re-suspended in fresh experimental media (10 ml). These cells were either used for the cellular viability assay, or re-seeded (1 ml) into the flask with 40 ml of culture medium and incubated at 37 ^{o}C in 5% CO_2.

6.2.2.4 Cellular viability assay

To determine the effect of the extract on cell survival, the trypsinized single-cell suspension was adjusted to 0.5 million cells/ml. This was accomplished by staining the cells with 2 mg/ml trypan blue (Sigma) in a 1:1 ratio, and then counting the number of cells with a haemocytometer. The cell suspension (90µl) was then plated out into the test and cell growth control wells; while experimental media (90 µl) was plated in the media and extract colour control wells. The media control was included to make sure the media was not contaminated, whereas the colour control was to account for the colour of the plant extract or possible plant-MTT interaction in the experiment. Two wells with 1% DMSO were included in the assay to test the effect of the extract solvent on the cells. The cells were then incubated for six hours at 37 ^{o}C in 5% CO_2 to allow them to adhere to the plastic surface of the 96 well plates (van Zyl *et al*., 2006). Thereafter, 10 µl of the prepared dilutions were added to the test wells and 10 µl experimental media to the cell growth control wells. The cells were further incubated for 44 hours at 37 ^{o}C in a 5% CO_2 humidified environment. After incubation, MTT (USB Corporation) (20 µl; 50 mg/ml solution in PBS) was added to the wells and incubated for 4 hours. Thereafter, 100 µl culture media was removed and DMSO (150 µl) added to stop the reaction and dissolve the formazan crystals. The plates were shaken on a plate reader for 2.5 minutes to ensure that all crystals were dissolved. Absorbance was measured at a test wavelength of 540 nm and reference 690 nm with a Labsystems iEMS Reader MF using the Ascent® software (version 2.4).

6.2.3 Data analysis

Percentage cellular viability was calculated with the two controls taken into account, using the following formula:

% Cellular viability =

$$\frac{\text{EXT treated cells Abs}_{(540\text{-}690)} - \text{mean Abs of EXT colour control}_{(540\text{-}690)} - \text{mean Abs of media control}_{(540\text{-}690)}}{\text{mean Abs of cell growth control}_{(540\text{-}690)} - \text{mean Abs of media control}_{(540\text{-}690)}}$$

where: EXT = extract and Abs = absorbance

The IC_{50} values (concentration causing 50% cell death) were generated using the Enzfitter® program (version 1.05) upon plotting log sigmoid dose-response curves. Each extract or positive control was plated in triplicate, as well as three separate experiments being performed for each extract. The results are presented as mean ± s.d. ANOVA and Student t-test were used to compare activity between the different plant parts, as well as to compare extract activity with the controls using Graphpad Prism® (version 4.0). A P value of less than 0.05 ($P<0.05$) was considered significant. To determine selectivity of the extracts for the parasite and the relative toxicity profile of the extracts, the safety index was calculated using the following formula:

$$\text{Safety index} = \frac{\text{Toxicity } (IC_{50})}{\text{Antimalarial activity } (IC_{50})}$$

A correlation between toxicity of the extracts and their antimalarial activity was determined using linear regression with a 95% confidence interval.

6.3 Results

The results of the cytotoxicity screening of the extracts are summarized in Figure 6.1. The IC_{50} values ranged from 2.228 ± 0.913 μg/ml to a concentration greater than 200 μg/ml. Of the 33 extracts tested, 54.54% were non-toxic to human kidney epithelial cells (IC_{50} >100 μg/ml). The least toxic of the extracts were *H. acutatum* (roots), G. *densa* (whole plant), *B. salicina* (bark), *T. phanerophlebia* (bark), *B. setifera* (leaves), *Z. mucronata* (fruit) and *B. setifera* (flower), which had IC_{50} values greater than 200 μg/ml (percent cell death = 78.71 ± 1.34, 77.58 ± 0.65, 70.84 ± 2.72, 68.98 ± 4.30, 68.71 ± 5.97, 55.68 ± 3.68 and 53.07 ± 3.80

respectively). The bark of *Z. mucronata* was the most toxic (IC_{50} = 2.23 ± 0.91 µg/ml), followed by the bark of *O. sphaerocarpa* (IC_{50} = 8.11 ± 2.80 µg/ml), leaves of *S. cordatum* (IC_{50} = 8.65 ± 1.41 µg/ml), fruit of *O. sphaerocarpa* (IC_{50} = 10.39 ± 1.05 µg/ml) and *P. meyeri* (whole plant) (IC_{50} = 14.63 ± 1.62 µg/ml).

The cytotoxic activity of all the extracts was significantly different (ANOVA: $P<0.001$) to that of chloroquine (IC_{50} = 296.96 ± 5.18 µg/ml), whilst others such as *T. phanerophlebia* (leaves), *D. cinerea* (leaves), *R. communis* (fruit, leaves, stem), *H. acutatum* (leaves) and *F. glumosa* (leaves) were not significantly different from quinine ($P>0.05$), with an IC_{50} value of 141.34 ± 22.08 µg/ml. It was generally noted that there is variability in toxicity among plant parts of the same species. However, there was no statistical significant difference in the toxicity between most of the parts in each species; with the exception of *R. communis* which had significant difference in the toxicity of the fruit, leaves and stem ($P<0.05$).

The bark of *B. salicina* was the most selective against the malaria parasites, with low toxicity compared to antimalarial activity, with a safety index of >26.99 (Table 6.1). The leaves of *T. phanerophlebia* also displayed selective toxicity with a safety index of 16.10. However, compared to chloroquine or quinine, the two plant parts were incomparable as both these antimalarial drugs are highly active and the plant parts are only crude extracts and not pure compounds.

There was no correlation between the cytotoxic properties of the extracts and their antimalarial activity (r^2 = 0.01529). Comparison between toxicity of the leaves, bark and fruit/flower showed more toxicity in the bark, followed by the fruit/flower and leaves (Figure 6.2). The IC_{50} values were significantly different from each other using the Student t-test when averaged ($P <0.05$).

6.4 Discussion

Fifty-four percent (54.54%) of the extracts tested had no cytotoxic properties (IC_{50} > 100 µg/ml) against human kidney epithelial cells, with most of the plants having variable toxicity among the plant parts tested. As an example, *Z. mucronata* showed the highest toxicity in the bark extract (IC_{50} = 2.23 µg/ml), whereas the fruit and leaves had lower toxicity (IC_{50} =

189.11 ± 8.62 and 117.82 ± 2.56 µg/ml), respectively (Table 6.1). This can be explained by the fact that plants and their parts exhibit different profiles of secondary metabolites at different stages as well as vegetative phases of their life (Miliauskas *et al*., 2004; Buenz *et al*., 2007). As an example, the UPLC chromatogram of *R. communis* varied among the different plant parts with the stem showing the least number of compounds (Figure 3.8). *Ozoroa spaherocarpa*, *S. cordatum* and *G. spatulifolia* were the only plants with general toxicity among all the plant parts.

R. communis has previously been reported to have toxic compounds such as ricin and *R. communis* agglutinin-60 from the seeds, and ricinine from the leaves. Ricin has been reported to inhibit eukaryotic protein synthesis by irreversibly binding to the 60S subunit and

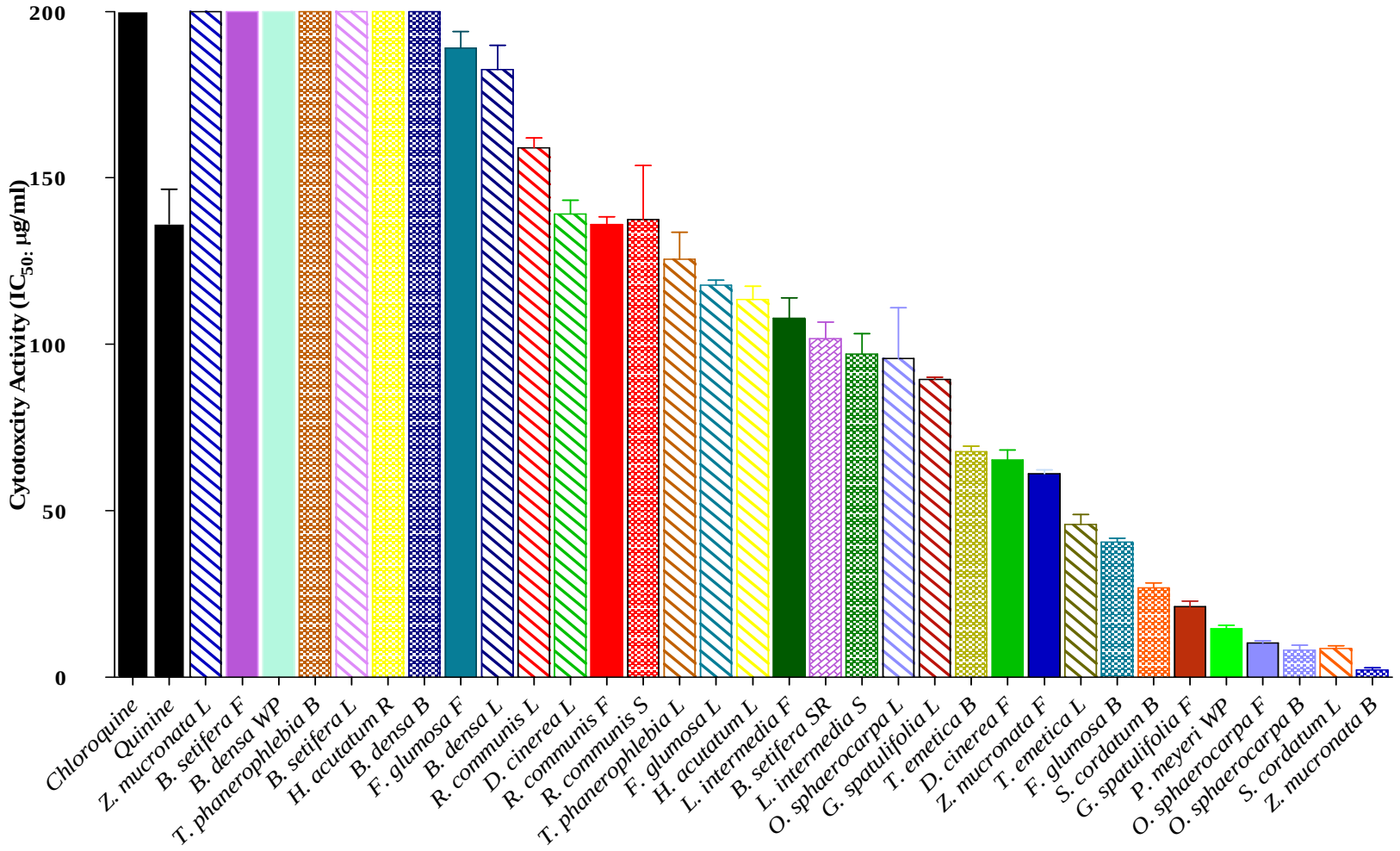


Figure 6.1: Cytotoxicity of plant extract in comparison with controls, on human kidney epithelial cells. Where: L = leaves, F = fruit, SR = stem/root, B = bark and WP = whole plant.

Table 6.1: *In vitro* cytotoxicity, antimalarial activity and safety index of extracts tested on human kidney epithelial cells, with percent cell death in parenthesis.

Scientific Name	**Plant part**	**Cytotoxicity IC_{50} (µg/ml) ± s.d.**	**Antimalarial activity IC_{50} (µg/ml) ± s.d.**	**Safety index**
Berkheya setifera	flower	>200 (53.07 ± 3.80%)	32.3 ± 0.74	(>6.19)
	stem/root	101.79 ± 8.42	15.12 ± 1.80	6.73
	leaves	>200 (68.71 ± 5.97%)	66.40 ± 4.19	(>3.01)
Breonadia salicina	leaves	182.66 ± 12.44	135.79 ± 10.75	1.35
	bark	>200 (70.84 ± 2.72%)	7.41 ± 1.88	(>26.99)
Dichrostachys cinerea	fruit	65.33 ± 5.00	26.51 ± 1.37	2.46
	leaves	139.19 ± 7.07	106.76 ± 9.64	1.30
Ficus glumosa	fruit	189.11 ± 8.62	117.54 ± 14.03	1.61
	leaves	117.82 ± 2.56	19.40 ± 2.20	6.07
	bark	40.56 ± 2.03	>200 (74.84 ± 3.95%)	(>0.20)
Gardenia spatulifolia	fruit	21.30 ± 2.67	124.90 ± 9.79	0.17
	leaves	89.46 ± 1.24	>200 (54.89 ± 4.84%)	(>0.45)
Guilleminae densa	whole plant	>200 (77.58 ± 0.65%)	162.32 ± 17.38	(>1.22)
Helichyrsum acutatum	leaves	113.53 ± 6.91	>200 (66.14 ± 7.69%)	(>0.57)
	roots	>200 (78.71 ± 1.34%)	>200 (76.67 ± 3.56%)	(>1)
Leonotis intermedia	flower	107.81 ± 10.78	55.51 ± 7.11	1.94
	stem	97.19 ± 10.50	25.98 ± 1.74	3.74
Ozoroa sphaerocarpa	fruit	10.39 ± 1.05	40.20 ± 8.54	0.26
	leaves	95.86 ± 26.27	54.33 ± 1.24	1.76
	bark	8.11 ± 2.80	45.29 ± 2.33	0.18
Priva meyeri	whole plant	14.63 ± 1.62	7.36 ± 0.85	1.99
Ricinus communis	fruit	136.11 ± 3.96	33.61 ± 2.86	4.05
	leaves	159.06 ± 5.00	23.60 ± 2.28	6.74
	stem	137.53 ± 28.06	52.73 ± 0.44	2.61
Syzygium cordatum	leaves	8.65 ± 1.41	32.61 ± 2.05	0.27
	bark	26.80 ± 2.54	6.70 ± 1.21	4.00
Terminalia phanerophlebia	leaves	125.64 ± 13.99	7.81 ± 1.82	16.10
	bark	>200 (68.98 ± 4.30%)	72.62 ± 3.40	(>2.75)
Trichilia emetica	leaves	45.95 ± 5.10	18.13 ± 3.55	2.53
	bark	67.83 ± 2.78	>200 (71.07 ± 7.78%)	(>0.34)
Ziziphus mucronata	fruit	>200 (55.68 ± 3.68%)	67.18 ± 7.40	(>2.98)
	leaves	61.17 ± 1.78	73.99 ± 1.47	0.83
	bark	2.23 ± 0.91	94.84 ± 1.47	0.02
Chloroquine		269.96 ± 5.18	0.052 ± 0.003	5191.56
Quinine		141.34 ± 22.08	0.13 ± 0.01	1087.23
Ciprofloxacin		>100 (67.59 ± 1.12%)	-	-
Amphotericin B		>100 (64.85 ± 1.68%)	-	-

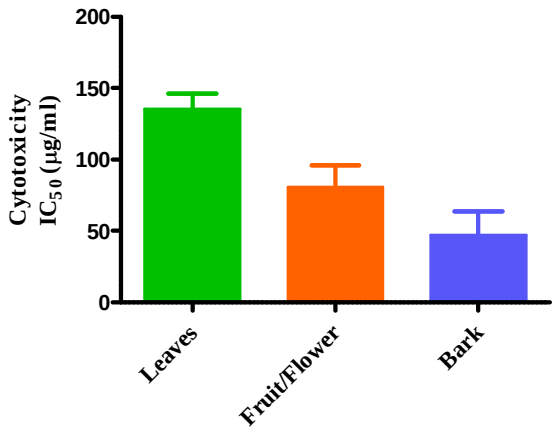


Figure 6.2: Comparison in toxicity between the different plant parts.

inactivating it (Balint, 1974; Lugnier *et al.*, 1977; Ferraz *et al.*, 1999). This study has however, shown non-toxicity of all the parts tested, with IC_{50} values greater than 100 µg/ml (Table 6.1). According to previous studies, ricinine, a neutral alkaloid might have a role in the inhibition of the mitochondrial respiratory chain. The MTT assay used in this study measures mitochondrial viability and therefore ricinine would be expected to be cytotoxic to the cells using this assay. It has also been reported that *R. communis* is highly toxic by causing both DNA and chromosomal aberrations (Ferraz *et al.*, 1999; Fennell *et al.*, 2004).

In contrast, a study conducted by Bessong *et al.*, (2005) found no cytotoxicity of the leaf methanolic extract against the HelaP4 cell line at concentrations as high as 400 µg/ml. These differences in toxicity can be due to the different cell lines or solvents used, as well as geographical variation since the plants were collected from different localities. Geographical variation in plant activities has been confirmed in *Salvia* spp and *Perlagonium* spp (Kamatou *et al.*, 2008; Lalli *et al.*, 2008). Castor oil from *R. communis* has reportedly been used to stimulate labour and also used as a mild laxative in clinical practice. It was also found to cause little or no skin irritation, had no sensitizer or photosensitizer properties, thus endorsed to be used in cosmetic products (Cosmetic Ingredient Review Expert Panel, 2007).

The leaves and fruit of *F. glumosa* were non-toxic (IC_{50} = 117.82 ± 2.56 and 189.11 ± 8.62 µg/ml, respectively) on the kidney epithelial cells, whereas the bark was toxic (IC_{50} = 40.56 ±

2.03 µg/ml) (Figure 6.1). Al-Fatimi *et al*. (2007) has shown non-toxicity of the fruit methanolic extract of *F. vasta* (IC_{50} = 980 µg/ml) against FL-cells (human amniotic epithelial cell line) using the neutral red uptake assay. Another study to report non-toxicity of the leaf ethanolic extract of *F. capensis* at concentrations greater than 50 µg/ml (Zirihi *et al*., 2005) as conducted on the human diploid embryonic lung cells (MRC-5) and rat myoblast-derived cells (L-6) using the MTT assay, thus confirming our results. Chiang *et al*. (2005) also reported toxicity of eight pentacyclic triterpenes possessing a carboxylic acid at C-28 isolated from the aerial parts of *F. microcarpa*, tested on human nasopharyngeal carcinoma HONE-1, oral epidermoid carcinoma KB and colorectal carcinoma HT29 cells using the methylene blue dye assay (IC_{50} values ranging between 0.5 to >10 µM).

T. phanerophlebia leaves showed acceptable toxicity levels (IC_{50} = 125.64 ± 13.99 µg/ml) and combined with its good antiplasmodial activity, it had the highest safety index recorded for the 33 extracts studied (Table 6.1). In contrast, a similar species *T. glaucenscens* showed high toxicity (IC_{50}: ~ 9 µg/ml) against human fibroblast cells (Mustofa *et al*., 2000). *Termianalia sericea* also showed significant toxicity against monkey kidney cells (IC_{50} = 24.00 µg/ml) (Tshikalange *et al*., 2005). A study conducted by Fyhrquist *et al*. (2006) on the toxicity of some *Terminalia* species, revealed varied toxicity of the genus against Hela cervical carcinoma cell line, MCF-7 breast cancer, PC 3 prostrate cancer, HOS osteosarcoma and T24 bladder cancer cells using both the Alamar Blue assay and flow cytometry assays with percent cell growth at 25 µg/ml ranging from 18.0 ± 0.24 to 110.0 ± 4.8%. The *Terminalia* species have been reported to possess many secondary metabolites such as saponins, flavones, flavonols, tannins and phenylpropanoids which are known to be cytotoxic and/or antimutagenic (Fyhrquist *et al*. 2006). The present study has also reported the presence of flavonoids in both the leaves and bark of *T. phanerophlebia*, with the bark having the higher content (33.33 mg RE/ g extract; Table 7.2) of flavonoids. Even though such high content of flavonoid has been reported in the bark, it was the least toxic to human kidney epithelial cells. This may mean that the types of flavonoids present are predominantly non-toxic or that the overall toxicity results from interactions within the individual components and this species of *Terminalia* may lack some of those compounds. *Terminalia* spp are traditionally used to treat a wide variety of illnesses such as diabetes, diarrhoea, gonorrohoea (roots and leaves), infected

wounds (powdered leaves), bilharzia (root decoction), fever, hypertension, cancer and bacterial infections (roots, stem bark and leaves) (Neuwinger, 1996).

The fruit and bark of *O. sphaerocarpa* were toxic as demonstrated in this study (Table 6.1). This correlates with a study by Rea *et al*. (2003), where they found cytotoxicity of the bark methanolic extract of a similar species *O. insignis*, tested against Hep-G2 (human hepatocellular carcinoma), MDA-MB-231 (human mammary adenocarcinoma) and 5637 MDA-MB-231 (human primary bladder carcinoma). The cytotoxicity was expressed as percent kill at 250 and 125 µg/ml; with 250 µg/ml resulting in 86.96 ± 4.83% and 92.30 ± 3.58% for Hep-G2 and MDA-MB-231, respectively. Whilst for the bladder carcinoma 125 µg/ml inhibited 91.56 ± 2.21% cell growth. The toxicity was attributed to the anacardic acid and ginkgoic acid components in *O. insignis*.

O. sphaerocarpa showed good antimicrobial activity (Table 4.1) against all the microorganisms tested. This general activity may be due to its general toxicity, resulting in non-specific cell death. The leaves, which showed less toxicity compared to the fruit or the bark, were also found to have lower antimicrobial activity (Table 6.1 and Table 4.1). It is interesting to note, though that the fruit of *O. sphaerocarpa* has no recorded medicinal use. Its use is only as a fruit, edible even by humans. The toxicity displayed in this study may be due to the fact that the flesh and the seeds were not separated during extraction and the toxicity may be due to the seeds. This could be because seeds, as agents for future, have defenses against predators to ensure that they are left untouched to germinate. These defenses are also in the form of toxins which make the seed unpalatable or indigestible (Salisbury and Ross, 1992; Stern, 1997). The *R. communis* seed oil has been reported to be quite toxic due to the presence of the phytotoxin ricin, which is found only in the seeds of this plant. In contrast, the fruit pericarp is used medicinally and does not contain ricin (Lugnier *et al*., 1977; Alternative Gardening, 2006). Another example is *Carica papaya* L. which has edible fruits, but the seeds contain benzyl isothiocyanate, which causes many toxic effects such as cell death via modification of mitochondrial function (Nakamura *et al*., 2002) and irreversible uterine tocolysis in rats (Adeyibi *et al*., 2003).

In this study it has been shown that the dominant toxicity of the fruits of *G. spatulifolia* and *D. cinerea* were probably due to the same reason in that the seeds were not separated from the fruit pericarp, whereas the seeds from the fruit of *R. communis* were separated and did not show toxicity (Table 6.1). *Ozoroa sphaerocarpa* has shown general toxicity among its parts and if the fruit is toxic when ingested, the compound taken is in small amounts which is different to the experimental condition where the use of many fruits and solvent may contribute to concentrating the toxic compound and thus result in the observed toxicity. It is also possible that the toxic compound is common in all parts of the plant. The UPLC chromatogram (Figure 3.7) showed a number of compounds common in all three fruit, leaves and bark extracts.

The bark of *Z. mucronata*, traditionally used for infections such as boils, chest problems and cough was also toxic to human kidney epithelial cells and has been shown to possess mutagenic properties using the Ames test on *Salmonella typhimurium* (Elgorashi *et al*., 2003).

S. cordatum was generally toxic in this study. Interestingly, it was found to have antigenotoxic effects exhibited by lowering the mutagen mitomycin C (MMC) in the micronucleus test (Fennell *et al*., 2004). The latter effect was observed by Verschaeve *et al*. (2004), where leaves of *S. cordatum* were found to have a protective effect against the genotoxic action of the chemical mutagen MMC on the *S. typhimurium* TA98 strain.

The flower, stem/root and leaves of *B. setifera* were non-toxic to the human kidney epithelial cells (Table 6.1). A similar species, *B. mantana* has been investigated for mutagenicity and negative results were observed using the Ames assay on *Salmonella typhymurium* (Reid et *al*., 2006). This ssupports the non-toxicity of the plant in general. In the same study, *Helichrysum* species (*H. simillimum*, *H. rugulosum* and *H. herbaceum*) showed positive mutagenicity results at concentrations ranging from 0.05 to 5 mg/ml. While in this study, no cytotoxicity of both the root and leaf extract of *H. acutatum* (IC_{50} >100 μg/ml) was observed.

L. intermedia (flower and stem) were not toxic with IC_{50} values of 107.81 ± 10.78 and 97.19 ± 10.50 μg/ml, respectively (Table 6.1). This is in agreement with a recent study conducted by Ah Kee *et al*. (2008), which showed no cytotoxicity (IC_{50} > 200 μg/ml) of the root methanolic

extract of *L. leonorus* on colon adenocarcinoma cell (HT-29), lung epithelial carcinoma (A-549), chronic myelogenous leukaemia (K562) and breast adenocarcinoma (MCF-7), with the exception of acute promyeloctic leukaemia (HL-60) (IC_{50} = 62.47 ± 3.31 μg/ml). A compound isolated from this plant, marrubiin also showed weak cytotoxic potential in the same study with ±10 to 25% inhibition of growth on the latter cell lines at 50 μg/ml. In contrast, *L. nepetifolia* was found to be toxic in the brine shrimp lethality assay with a LC_{50} of 19.8 μg/ml (David *et al*., 2007).

The fruit and leaves of *G. spatulifolia* were found to be toxic in this study (Figure 6.1), which is in agreement with previous studies that have shown that a similar species *G. volkensii* causes DNA damage and chromosomal aberrations using the micronucleus and alkaline comet assays (Taylor *et al*., 2003; Verschaeve *et al*., 2004). *Gardenia* yellow colour from the fruit of Gardenia species was shown to induce hepatotoxicity in male Wistar rats due to geniposide, which is an iridoid (Yamano *et al*., 1988), and Ozaki *et al*. (2002) has also reported genotoxicity of geniposide. Cytotoxic compounds have been reported for the leaf and twig constituents of *G. tubifera* (Reutrakul *et al*., 2004). Coronalolide methyl ester and coronalolide, isolated from *G. coronaria* and *G. sootepensis* were similarly reported to be cytotoxic when tested against a hormone-dependant breast cancer cell line (ZR-75-1) (Silva *et al*., 1997).

The leaves of *D. cinerea* did not show cytotoxicity (IC_{50} value = 139.19 ± 7.07 μg/ml) whereas the fruit was toxic (IC_{50} value = 65.33 ± 5.00 μg/ml) to human kidney epithelial cells (Figure 6.1). In a previous study, the tannins isolated from the root of *D. cinerea* were reported to possess antibacterial properties (Banso and Adeyemo, 2007). In agreement, this study has reported good antimicrobial activity in *D. cinerea* with MIC range of 0.25 to 0.67 mg/ml (Table 4.1), indicating a more selective inhibitory / toxic effect towards micro-organisms than eukaryotic cells.

The leaves and bark of *T. emetica* were both cytotoxic with IC_{50} values of 45.95 ± 5.10 and 67.83 ± 2.78 μg/ml, respectively. In their review, Fennell *et al*. (2004) reported that *T. emetica* is highly toxic and causes both DNA damage and chromosomal aberrations and this was previously reported by Elgorashi *et al*. (2003) and Taylor *et al*. (2003).

G. densa (whole plant) and *B. salicina* (leaves and bark) were non toxic, whilst *P. meyeri* (whole plant) was toxic to kidney epithelial cells (Figure 6.1). This is the first documentation on the cytotoxicity of these plants as a comprehensive literature search yielded no previous toxicological work on them.

Overall, there was no correlation between the cytotoxic properties of the extracts and their antimalarial activity (r^2 = 0.01529), indicating that for the majority of the plant extracts the cytotoxic properties are not related to the possible mechanism by which the extracts are inhibiting plasmodial growth. Caution should still be taken when administering these extracts as some do possess a high cytotoxic activity. The bark was more toxic when compared to the other plant parts, followed by the fruit/flower and lastly the leaves. This may possibly be due to the presence of tannins in the bark which are toxic depending on the degree of polymerization (Saha and Kaviraj, 1996; Makkar *et al*., 2007).

The discrepancies in toxicity emphasize the need for rigorous testing of traditional medicinal preparations before they are prescribed as medicines. One assay may give some beneficial effects of a particular extract, whereas another one may show undesirable effects, emphasizing the need to screen various cell lines using various assays. As with all conventional drugs, traditional medicines will have side effects as well as the desired effects. They should, therefore, be fully investigated for both short-term (acute poisoning), long-term (mutagenic) and teratogenic effects.

Moreover, issues of herb-drug interactions should be properly investigated. Medicinal plants, if exerting a clinical effect, may also interact with concurrently used pharmaceutical medicines, for example, the common self-administered use of St John's wort taken concurrently with the combined contraceptive pill, digoxin, HIV protease inhibitors, selective serotonin reuptake inhibitors, theophylline and warfarin, has produced severe interactions (Heinrich *et al*., 2004).

The current study demonstrates potential toxicity of some of the plants investigated. However, it is important to note that *in vitro* cytotoxicity is not always indicative of *in vivo* toxicity

(Cremin and Smith, 2002). Therefore, it would be unwise to conclude that the plants studied will have toxic effects on humans when administered as traditional medicines. Plant extracts, like most drugs will be subjected to various metabolic processes in humans which may result in more or less toxic metabolites (Klaassen, 2001). The study only indicates that the plants could be toxic to humans, but for more conclusive work, it should be expanded to include tests on other cell lines, covering all major systems as well as conducting *in vivo* and clinical investigation before conclusions are made about their toxicity to humans.

CHAPTER 7: ANTI-OXIDANT ACTIVITY AND TOTAL PHENOLIC/FLAVONOID CONTENT

7.1 Introduction

7.1.1 Oxidation of biological systems and anti-oxidants

Oxidation is a process in which a loss of electrons occurs. It is essential to many living organisms for the production of energy to fuel biological processes (Turkoglu *et al*., 2006). Active oxygen, either in the form of superoxide, hydrogen peroxide, hydroxyl radical or singled oxygen, is a product of normal metabolism. Oxidants have been implicated in the onset and/or propagation of many human diseases such as atherosclerosis, cardiac and cerebral ischaemia, rheumatic and pulmonary diseases (Quettier-Deleu *et al*., 2000).

7.1.2 Sources of reactive oxygen species

Reactive oxygen species are derived from both endogenous and exogenous sources. Exogenous sources include amongst others; UV irradiation, γ-irradiation, ultrasound, food, drugs, pollutants, xenobiotics and toxins; while endogenous sources are cells (neutrophils, monocytes, macrophages, eosinophils), direct producing reactive oxygen species (ROS) enzymes such as nitric oxide (NO) synthase, indirect-producing ROS enzymes (xanthine oxidase), metabolism and diseases (mental disorders, ischaemic processes) (Ames *et al*., 1993; Halliwell, 1994; Kohen and Nyska, 2002).

7.1.3 Physiological functions of free radicals

A free radical is any atom or molecule that contains one or more unpaired electrons. These unpaired electrons render the molecule very reactive and once generated a free radical is capable of propagating by being involved in chain reactions with other less reactive species. Examples of free radicals include hydroxyl ($OH^{\cdot}$), superoxide ($O_2^{\cdot}$), nitric oxide ($NO^{\cdot}$), nitrogen dioxide ($NO_2^{\cdot}$) and peroxyl ($ROO^{\cdot}$) (Halliwell and Gutteridge, 1989; Bahorun *et al*., 2006). Free radicals act as messengers for signal transduction and also affect gene expression**.** Nitric oxide has been identified as a signaling molecule since it has the ability to cross cell membranes thereby transmitting signals to other cells. It also regulates transcription factor activities and other determinants of gene expression. Hydrogen peroxide and superoxide have similar effects on the cell (Nordberg and Arnèr, 2001).

Free radicals are also useful in the body's defence against infection. When phagocytes are activated, they produce reactive oxygen species in amounts that are enough to kill invading microorganisms (Thomas *et al.*, 1988). During a microbial invasion, ROS are produced by the NADPH oxidase complex that converts oxygen to superoxide, which is in turn reduced in the phagosome by superoxide dismutase (SOD) to hydrogen peroxide that is also converted to hypochlorous acid by myeloperoxidase. Hypochlorous spontaneously forms hydroxyl radical by the Fenton reaction. Hypochlorous and the hydroxyl radical are highly toxic and cause bacterial DNA damage (Nordgerg and Arnèr, 2001; Kohen and Nyska, 2002).

7.1.4 Deleterious effects of free radicals

Free radicals also attack biological molecules, leading to cellular or tissue damage (Figure 7.1). This damage results when the mechanism of anti-oxidant protection becomes unbalanced due to factors such as smoking, ionization radiation, certain pollutants, organic solvents, normal aerobic respiration, stimulated polymorphonuclear leukocytes and macrophages (Büyükokuroğlu *et al.*, 2002; Gülçin *et al.*, 2004; Turkoglu *et al.*, 2006; Tiwari and Tripathi, 2007). Diseases such as carcinogenesis, mutagenesis, ageing, arteriosclerosis, rheumatoid arthritis, malaria, gastric ulcer and AIDS have all been associated with the production of oxygen-derived free radicals and reactive oxygen species (Covacci *et al.*, 2001; Zhu *et al.*, 2002; Büyükokuroğlu *et al.*, 2002; Gülçin *et al.*, 2004; Turkoglu *et al.*, 2006). Free radicals are known to attack and cause specific damage to certain vital systems in the body. These may be damage to DNA, lipids and proteins.

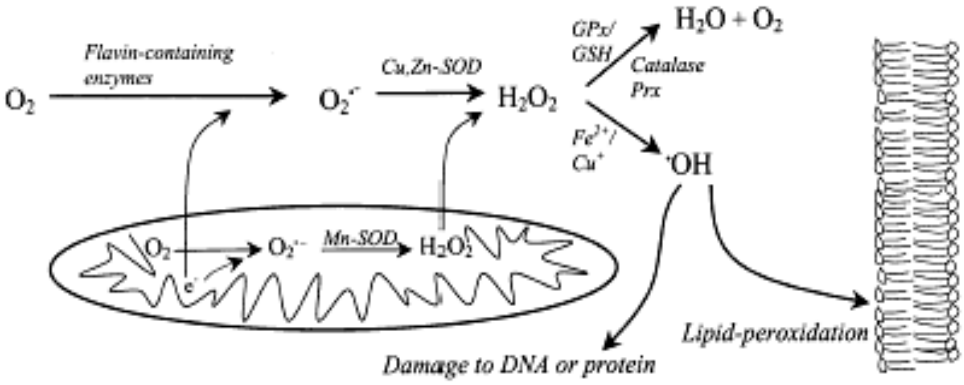


Figure 7.1: Simplified scheme of oxidative and anti-oxidative systems in cells, where GSH: glutathione; GPx: glutathione peroxidase; Prx: peroxiredoxins (Nordberg and Arnér, 2001).

7.1.4.1 Deoxyribonucleic acid

ROS can interact with DNA and cause several types of alterations including DNA cleavage, DNA protein cross links, oxidation of purines, damage to the deoxyribose sugar and damage to the DNA repair system. If the DNA damage repair system is unable to fix the damage immediately, mutations may result, which may explain the prevalence of cancer in individuals exposed to oxidative stress. Aging has also been attributed to mitochondrial DNA damage (Nordgerg and Arnèr, 2001; Kohen and Nyska, 2002).

7.1.4.2 Lipids

Lipid peroxidation is defined as the net result of any free radical attack on membranes and other lipid constituents present in the body. It can either be enzymatic (Fe^{2+}/NADPH) or non-enzymatic (Fe^{2+}/ascorbic acid) (Tiwari and Tripathi, 2007). The multiple bonds of fatty acids are good targets for ROS. Such oxidation is also involved in the generation of atherosclerotic plaques. Plaque formation is also involved in the aetiology of cardiovascular diseases (Halliwell, 1994). The damage to lipids occurs in three stages. The first stage is initiation, which involves the attack of a reactive oxygen metabolite capable of abstracting a hydrogen atom from a methylene group in the lipid. Following the abstraction of the hydrogen, the remaining fatty acid radical retains one electron and is stabilised by rearrangement of the molecular structure to form a conjugated diene. This radical can react with oxygen to form $ROO^{\cdot}$ during the propagation stage. The radicals are themselves capable of abstracting another hydrogen atom from a neighbouring fatty acid molecule, which leads to the production of the fatty acid radical that undergoes the same reactions. $ROO^{\cdot}$ becomes a lipid hydroperoxide by rearrangement. This can lead to a chain of reactions resulting in the peroxidation of all the unsaturated lipids in the membrane (Kohen and Nyska, 2002; Halliwell and Gutteridge 1989). Iron stimulates lipid peroxidation by the Fenton reaction and also accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can also abstract hydrogen and perpetuate the chain reaction of lipid peroxidation (Halliwell, 1994).

7.1.4.3 Proteins

Proteins, as major constituents of membranes, can also serve as targets for oxidative damage, especially those containing sulphur or selenium residues. Oxidative damage to proteins results in peroxidation, damage to specific amino acid residues, changes in their tertiary structure,

degradation and fragmentation. All these lead to less active enzymes, and non-functioning proteins (Stadtman and Berlett, 1998).

7.1.5 Transition metal-induced oxidative damage

Transition metals, especially copper and iron, which are present in relatively high concentrations in the body, play a very important role in the production of free radical species. Interaction between iron and H_2O_2 produces the OH^- radical via the Fenton reaction (Figure 7.2A), which is recognised as one of the most important explanations to the oxidative damage occurring in biological environments. At the physiological pH, most of the iron is oxidised and attached to biological chelates in the form of Fe^{3+}. To take part in the Fenton reaction, it must first be converted to its reduced form, Fe^{2+} by superoxide radicals (Figure 7.2B). Due to the fact that metals are usually bound to either protein or membranes, their involvement in the Fenton reaction and the production of the OH^- radical can have deleterious effects on the body, since the OH^- radical is in close proximity to biological sites (Kohen and Nyska, 2002).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^- \qquad (A)$$

$$Fe^{3+} + O_2^{-} \rightarrow Fe^{2+} + O_2 \qquad (B)$$

Figure 7.2 Fenton reaction (Kohen and Nyska, 2002).

7.1.6 Anti-oxidants

Anti-oxidants can protect the human body from the effects of free radicals and ROS (Gülçin *et al*., 2005). The most commonly used synthetic anti-oxidants are butylated hydroxyanisole, butylated hydroxytoluene, propyl gallate and tert-butylhydroquinone (Sherwin, 1990). Unfortunately, these anti-oxidants have been linked to liver damage and carcinogenesis in laboratory animals (Grice, 1988; Wichi, 1988). Natural anti-oxidants can be divided into two major types; enzymatic anti-oxidants and low molecular weight anti-oxidants and all play a vital role in protecting the body against free radical damage. The enzymatic anti-oxidants are Cu-Zn-SOD, Mn-SOD, glutathione peroxidase and catalase (Figure 7.3). Low molecular weight anti-oxidants include ascorbic acid (vitamin C), tocopherols (vitamin E), carotenoids, flavonoids, tannins and glutathione. Cu-Zn-SOD is responsible for the conversion of superoxide anion into hydrogen peroxide and is found in the cytoplasm and mitochondrial

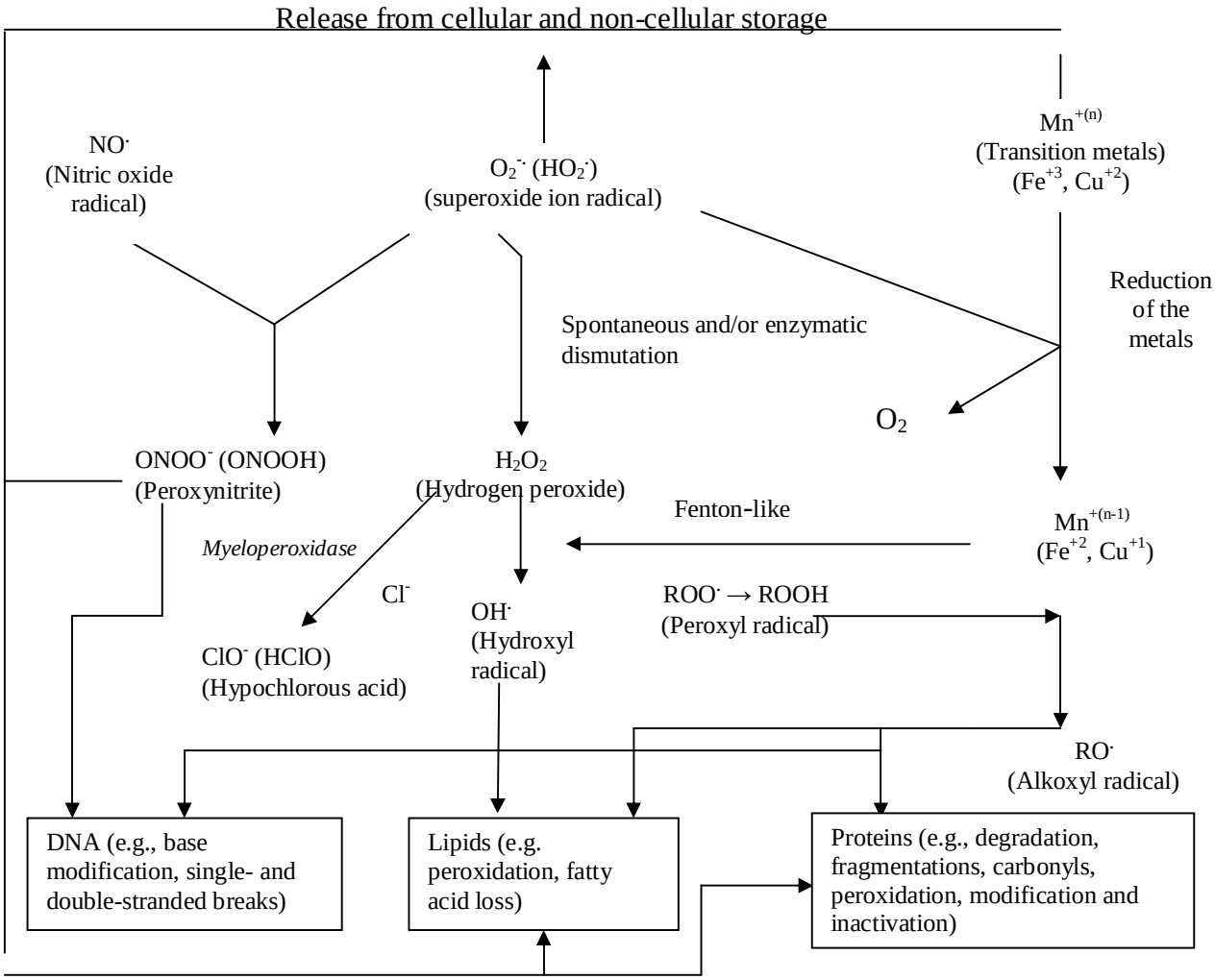


Figure 7.3: Schematic diagram representing a series of reactions resulting in reactive oxygen species – induced oxidative damage (Kohen and Nyska, 2002).

intermembrane space. Mn-SOD is found in the mitochondrial matrix and has the same function as Cu-Zn-SOD. Catalase catalyses the dismutation of hydrogen peroxide to oxygen and water. Glutathione peroxidase is responsible for the reduction of hydrogen peroxide and hydroperoxides that utilizes reduced glutathione as a hydrogen donor (Mau *et al.*, 2002, McCune and Johns, 2002; Turrens, 2004).

The overall effect of anti-oxidants has been explained by the restoration of the redox equilibrium disturbed by the causative factors mentioned above and thus diminishing the damage on cellular structures (Ames *et al.*, 1993).

Various mechanisms have been proposed to contribute to the overall effect of anti-oxidants, such as prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging (Diplock, 1997; Oktay *et al*., 2003). Plants used in traditional medicine have characteristically been shown to possess anti-oxidant activity (van Vuuren *et al*., 2006).

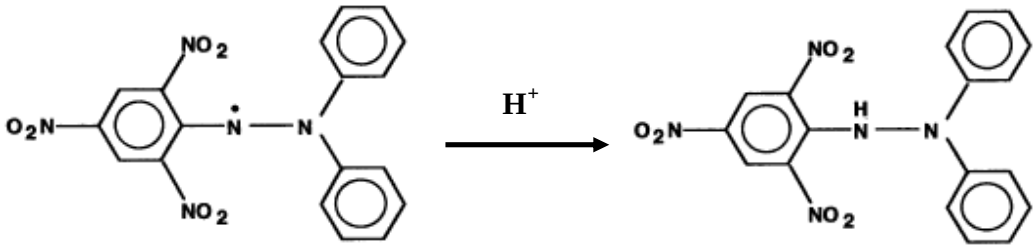
7.1.7 Traditional medicines as a source of anti-oxidants

Medicinal plants are known to contain large quantities of anti-oxidants such as phenolic acids, flavonoids and tannins, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Škerget *et al*., 2005; Djeridane *et al*., 2006; van Vuuren *et al*., 2006). The use of such species as *Aspalathus linearis* (rooibos), various species of *Cyclopia* (honeybush) and *Athrixia phyllicoides* (bush tea) as a herbal tea, date back to the 1900s in South Africa. These plants have been researched and various degrees of anti-oxidant activity have been reported (Joubert *et al*., 2008). The anti-oxidant activity amongst them has been attributed to their flavonoid, tannin and phenolic content (Joubert *et al*., 2008).

7.2 Materials and Methods

7.2.1 DPPH free radical-scavenging activity

Anti-oxidant properties and radical-scavenging activities are very important in biological systems due the deleterious effects of free radicals. Diphenylpicrylhydrazil (DPPH) has been widely used to evaluate the free radical-scavenging effectiveness of various anti-oxidant substances. $DPPH^{\cdot}$ is a stable free radical (Figure 7.4A) with a characteristic absorption band at 517 nm. Anti-oxidants donate protons to this radical resulting in the production of a non-radical form DPPH-H (Figure 7.4B), with a decrease in absorption at the characteristic wavelength (McCune and Johns, 2002; Molyneux, 2004; Gülçin *et al*., 2005; Turkoglu *et al*., 2006).



A: Diphenylpicrylhydrazyl (free radical) B: Diphenylpicrylhydrazine (nonradical)

Figure 7.4: DPPH· free radical with an unpaired electron (A) and DPPH-H, which is non-radical, has a hydrogen atom bound to the lone pair (B) (Molyneux, 2004).

7.2.1.1 Protocol

7.2.1.1.1 Microtitre plate method

(i) Preparation of DPPH

DPPH· (96.16 µM) (Sigma-Aldrich) was prepared in HPLC grade methanol. DPPH· is light sensitive, therefore the container was wrapped in foil, stored at +4 ^{o}C and used within a week of preparation.

(ii) Preparation of extract / control

The crude extract or ascorbic acid (10 mg/ml) was prepared in DMSO (Saarchem) and stored at -20 ^{o}C. For experimental purposes, this solution was diluted 1:20 to prepare the starting concentration of 500 µg/ml.

(iii) Preparation of microtitre plates

In a 96-well microtitre plate, 50 µl of the 500 µg/ml solution was plated in triplicate wells for the test and control wells (Figure 7.5). 200 µl DPPH· or HPLC grade methanol (Rochelle Chemicals) was added to the test and control wells, respectively. The final concentration of the extract in each well was 100 µg/ml, that is, a five-fold dilution from 500 µg/ml. DMSO (50 µl) and DPPH· (200 µl) were included for 100% absorbance. A blank control containing 50 µl DMSO and 200 µl methanol was also included as a negative background control. Upon addition of DPPH·, the reaction mixture was allowed to stand for 30 minutes in the dark at room temperature before the absorbance was read at 550 nm on a Labsystems Multiskan RC spectrophotometer using the Genesis® (Version 3.03) program. The radical scavenging potential of the plant extract was then calculated using the formula in Figure 7.6.

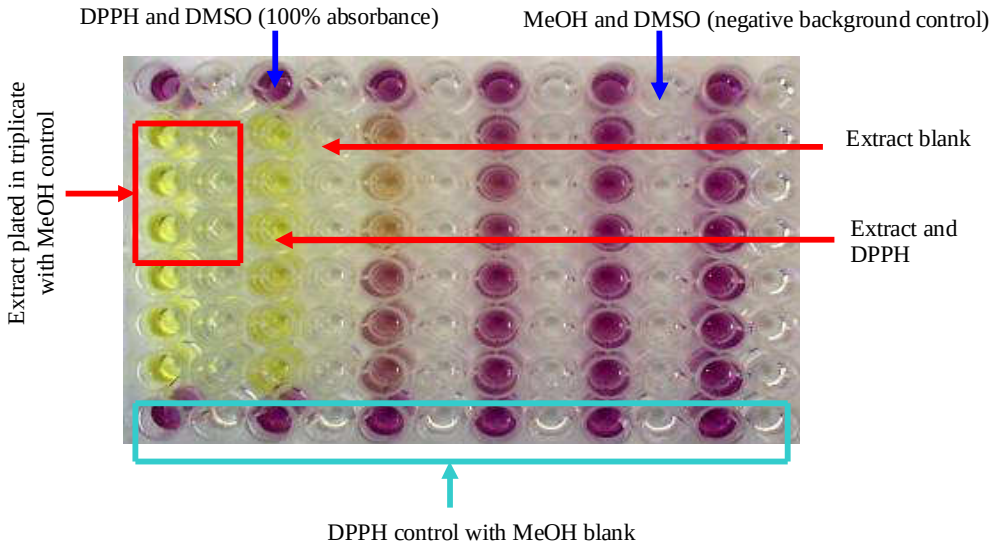


Figure 7.5: Plate layout used for determining DPPH· radical scavenging activity.

$$\% \text{ Decolourisation} = \frac{(\text{Av DPPH}\cdot \text{ control} - \text{Av methanol blank}) - (\text{Av extract} - \text{Av extract blank})}{(\text{Av DPPH}\cdot \text{ control} - \text{Av methanol blank})} \times 100\%$$

where:

Av DPPH· Control = mean absorbance of control for 100% absorbance

Av methanol blank = mean absorbance of methanol blank

Av extract = mean absorbance of extract

Av extract blank = mean absorbance of extract colour control

Figure 7.6: Equation used to determine percent (%) free radical scavenging activity of plant extracts.

This constituted the initial screening for DPPH· scavenging activity. Extracts that possessed DPPH· radical scavenging activity of less than 50% at 100 µg/ml were considered to have poor DPPH· radical scavenging activity. An extract that exhibited over 50% DPPH· scavenging activity (decolourisation) was serially diluted in DMSO and the various concentrations tested as previously described. A concentration giving 50% decolourisation (IC_{50}) was then calculated by plotting a log sigmoid dose-response curve using the Enzfitter® program. Ascorbic acid (Vitamin C) was used as a positive control.

7.2.1.1.2 High performance thin layer chromatography (HPTLC) method

The DPPH$^{\cdot}$ free radical scavenging activity of the most active extracts (IC_{50} value < 100 µg/ml) was also evaluated using an HPTLC method whereby extracts (10 mg/ml in methanol) were spotted on HPTLC plates and developed as described in Section 3.2.1. Thereafter, the plate was dipped in the DPPH$^{\cdot}$ radical (6.65 mM) in HPLC grade methanol, followed by air-drying at room temperature. Free radical scavenging activity was observed as decolourised (yellow) areas on the HPTLC plate.

7.2.2 Metal chelating activity

Iron is a very important biological molecule and is required by eukaryotic cells for survival, proliferation and as a constituent of haemoglobin and other haemoproteins (Hentze *et al*., 2004). Unfortunately, though important, iron overload can have damaging effects to lipid membranes. Chelating agents have been reported to be effective secondary anti-oxidants due to the fact that they reduce the redox potential thus stabilising the oxidized form of the metal ion (Gülçin *et al*., 2005). Ferrozine forms complexes with Fe^{2+} (Fe^{2+}-ferrozine) in a quantitative manner, with a stoichiometric ratio of 1:3. Upon addition of chelating agents, the complex formation is disrupted and the red colour of the complex fades away. Measuring the amount of colour reduction allows for the estimation of the chelating activity of the co-existing chelator. Reduction of the colour intensity means that the chelator captures the ferrous ion before ferrozine thus reducing the absorbance at the test wavelength (Dinis *et al*., 1994; Oktay *et al*., 2003; Gülçin *et al*., 2004; Gülçin *et al*., 2005; Sarradin *et al*., 2005; Zhan *et al*., 2006; Senevirathne *et al*., 2006).

7.2.2.1 Preparation of ferrozine

A 1.2 mM stock solution of ferrozine (3-[2-pyridyl]-5,6-diphenyl-1,2,4-triazine-4,4´-disulfonic acid·sodium-salt; Fluka) was prepared in MilliQ™ water and stored at +4 ^{o}C until required.

7.2.2.2 Preparation of 5% ammonium acetate

A 5% (w/v) ammonium acetate (Fluka) solution was prepared in MilliQ™ water and stored at +4 ^{o}C until required.

7.2.2.3 Preparation of $FeCl_2$

The $FeCl_2$ solution (0.48 mM; Fluka) was prepared fresh for each experiment in 5% ammonium acetate to prevent oxidation of the Fe^{2+} ions upon standing.

7.2.2.4 Wavelength scan for the ferrozine-iron complex

To determine the absorbance maxima of the ferrozine-iron complex, 20 µl DMSO, 30 µl $FeCl_2$ and 100 µl ferrozine were mixed and incubated for 10 minutes in the dark at room temperature. Thereafter a wavelength scan was done on a Lambda 25 Perkin Elmer UV/VIS spectrometer, using UV WinLab Version 2.85.04, between 700 and 400 nm. An absorbance maxima was found at 565.08 nm (Appendix E; Figure E1), therefore the method was adapted to a microtitre plate and ran absorbance measurements at 550 nm, which was the nearest available filter to the wavelength maxima.

7.2.2.5 Microtitre plate method

A stock solution of 10 mg/ml of crude extract was used. From this stock, a working solution of 750 mg/ml was prepared in DMSO.

Extract (20 µl) was plated out in a 96-well microtitre plate in triplicate wells. Two control wells of the same extract were also plated; a colour control well for extract colour (EC) and a control for the colour reaction between extract and iron (EF). In total, five wells of the same extract were plated on the microtitre plate (Figure 7.7). $FeCl_2$ (30 µl), extract (20 µl) and (100 µl) were plated in the EF well, whilst ammonium acetate (30 µl), extract (20 µl) and water (100 µl) were added to the EC control wells (Figure 7.7) and incubated for 10 minutes in the dark at room temperature. For 100% colour or complex formation, only DMSO (20 µl), $FeCl_2$ (30 µl) and ferrozine (100 µl) were plated in 8 wells, with three blanks included for the DMSO, ferrozine and $FeCl_2$. Absorbance was measured at 550 nm on a Labsystems Multiskan RC spectrophotometer using the Genesis® (Version 3.03) program. EDTA (Rochelle Chemicals) at the same concentration as Fe^{2+} in MilliQ™ water (0.48 mM) was used as a positive control. The metal chelating activity of the extracts was calculated using the formula in Figure 7.8.

The concentration required to reduce the Fe^{2+}-ferrozine complex colour by 50% (IC_{50}) was obtained by plotting the concentration of extract against % metal chelating activity and obtaining log sigmoid dose-response curves using the Enzfitter® program.

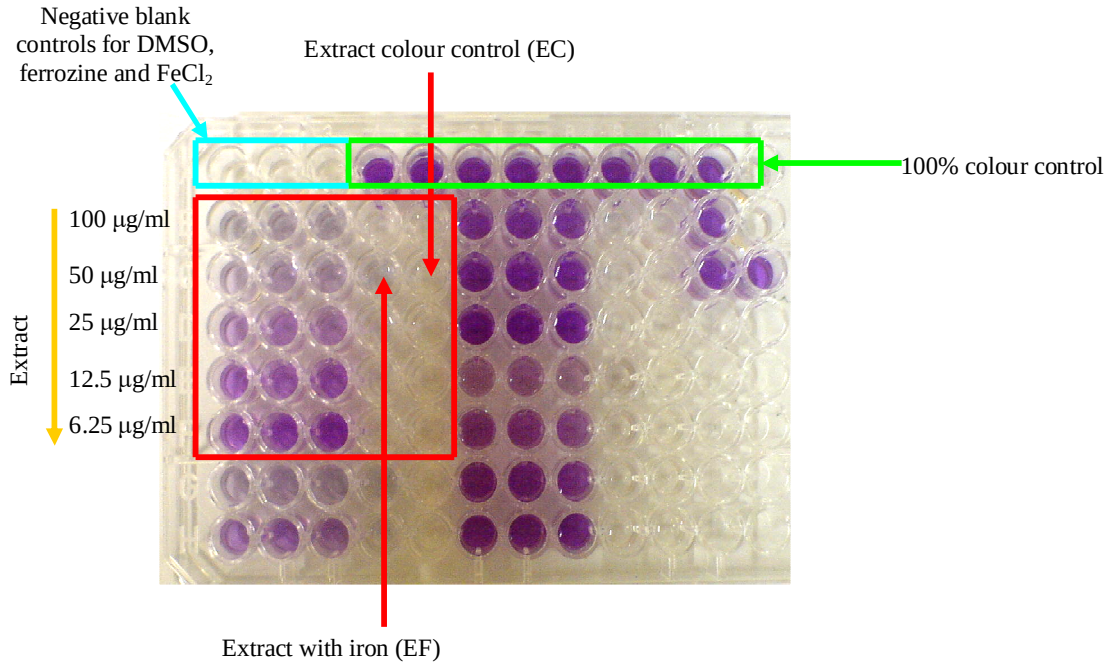


Figure 7.7: Plate layout for metal chelating activity.

$$\text{Metal chelating activity (\%)} = \frac{(\text{Av control} - \text{Ab blank}) - (\text{Ab extract} - \text{Ab extract blank})}{(\text{Av control} - \text{Av blank})} \times 100\%$$

where:

Av control = mean absorbance of control for 100% ferrozine binding

Ab blank = mean absorbance of all blanks (DMSO, ferrozine and $FeCl_2$)

Ab extract = absorbance of extract

Ab extract blank = absorbance of extract colour controls (EC and EF)

Figure 7.8: Equation used to calculate percent (%) metal chelating activity of extracts.

7.2.3 Inhibition of lipid peroxidation

Inhibition of lipid peroxidation was measured using the ferric thiocyanate method described by Gülçin *et al*., 2005; Zhan *et al*., 2006) where linoleic acid and plant extract are incubated at 37 ^{o}C for 48 hours in the dark. Thereafter, the mixture is reacted with ferrous chloride and ammonium thiocyanate. This method is based on the fact that during linoleic acid oxidation,

peroxides are formed. These compounds oxidize ferrous ions to ferric ions, which in turn form a complex with thiocyanate resulting in absorbance maxima at 500 nm. In the presence of an anti-oxidant, the complex formation is disrupted resulting in a decrease in absorbance. This disruption can be achieved through one or two of four mechanisms:

a) Preventing first-chain initiation by scavenging for radicals such at the hydroxyl radical.
b) Decomposing peroxides by converting them to non-radical forms.
c) Chain breaking, that is, to scavenge the intermediate radicals such as the peroxyl radical.
d) Binding metal ions such that they do not form chain initiating species (Halliwell and Gutteridge 1989).

7.2.3.1 Preparation of sodium phosphate buffer

The 0.2 M sodium phosphate buffer was prepared by dissolving 2.76 g $NaH_2PO_4.H_2O$ (Merck) in 100 ml MilliQ™ water (solution A), and 7.12 g $Na_2HPO_4.2H_2O$ (Merck) in 200 ml MilliQ™ water (solution B). To make the buffer with a pH of 7.0, 78 ml of solution A was mixed with 122 ml solution B. The pH of the buffer was confirmed using a Beckman 40 pH meter. This solution was sterilized through a 0.22 µm filter and stored at +4 ^{o}C until required.

7.2.3.2 Preparation of ammonium thiocyanate

A 0.3 g/ml (w/v) solution of ammonium thiocyanate (Saarchem) was prepared in MilliQ™ water, stirred until dissolved and thereafter stored at room temperature.

7.2.3.3 Preparation of ferrous chloride

The 20 mM stock solution of ferrous chloride tetrahydrate was dissolved in 50 ml 3.5% (v/v) hydrochloric acid in MilliQ™ water and stored in a glass container covered with foil at room temperature. This solution was prepared fresh for each experiment.

7.2.3.4 Preparation of linoleic acid emulsion

Linoleic acid (0.0568 g; Fluka) along with Tween-20 (0.0568 g; Sigma) were mixed and the volume made up to 10 ml with sodium phosphate buffer (pH 7.0). The solution was prepared fresh for each experiment.

7.2.3.5 Wavelength scan for ferric ions–thiocynate complex

To determine the absorbance maxima of the ferric ions–thiocynate complex, the protocol using DMSO, as outlined in Section 7.2.3.6 was used. Thereafter a wavelength scan was done on a Lambda 25 Perkin Elmer UV/VIS spectrometer, using UV WinLab Version 2.85.04, between 700 and 300 nm. An absorbance maxima was found at 481.04 nm (Appendix E; Figure E2).

7.2.3.6 Determination of lipid peroxidation

The extract (1 mg/ml) or DMSO for control (50 µl) was reacted with 250 µl linoleic acid emulsion and 200 µl sodium phosphate buffer in glass tubes covered with foil. This mixture was vortexed and incubated at 37 ^{o}C for 48 hours in the dark. Thereafter, 20 µl of the reaction mixture was reacted with 75% ethanol (900 µl; Rochelle Chemicals), 40 µl ammonium thiocyanate and 40 µl ferrous chloride. After stirring the reaction mixture for 5 minutes, absorbance was measured at 500 nm using a Philips PU8700 UV/Visible spectrophotometer. This was wavelength maxima was chosen due to the availability of a filter closest to the wavelength maxima found after the wavelength scan. Trolox™ (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Aldrich) and ascorbic acid (Saarchem), at the same concentration as the extract (prepared in DMSO), were used as positive controls. Inhibition of lipid peroxidation was calculated using the formula in Figure 7.9.

$$\% \text{ Inhibition} = (A_0 - A_1) / A_0 \text{ X } 100$$

where:

A_0 = absorbance of control

A_1 = absorbance of extract or positive control

Figure 7.9: Equation for determining inhibition of lipid peroxidation by the extracts.

7.2.4 Phenolic and flavonoid content

Phenols are important components of plants, found both in edible and non-edible plants. Plant phenolics are divided into the following main groups; phenolic acids that are hydroxylated derivatives of benzoic acid, phenolic acids derived from cinnamic acid and glycosidic phenylpropannid esters. Flavonoids on the other hand are a group of natural benzo-γ-pyran derivatives and occur as aglycones, glycosides and methylated derivatives. The latter

have been used for centuries as an active ingredient to treat human diseases, and have been shown to be anti-carcinogenic, anti-inflammatory, inhibit lipid peroxidation in meats, fish oil and lard (Havsteen, 1983; Chen *et al*., 1996).

Phenolic constituents, such as flavonoids, phenolic acids, diterpenes and tannins are especially worthy of notice due to their high anti-oxidant activity. They have been reported to eliminate radicals due to their hydroxyl groups (Hatano *et al*., 1989), whose position on the aromatic rings has an effect on the anti-oxidant activity of flavonoids (Škerget *et al*., 2005). Their redox properties allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers, and in addition, possess a metal chelating potential (Shahidi *et al*., 1992; Rice-Evans *et al*., 1996). A highly positive relationship exists between total phenols and anti-oxidant activity (Gülçin *et al*., 2004) and according to Tanaka *et al*., (1998), ingestion of up to 1.0g polyphenolic compounds on a daily basis has inhibitory effects on mutagenesis and carcinogenesis.

7.2.4.1 Phenolic content

The concentration of phenols in the extracts was measured using the oxidizing agent Folin-Ciocalteu reagent by UV spectrophotometry, which is based on a colorimetric oxidation/reduction reaction. A standard curve was prepared using gallic acid and the total phenolic content expressed as gallic acid equivalent (Slinkard and Singleton, 1977; Škerget *et al*., 2005).

7.2.4.1.1 Preparation of sodium carbonate

Sodium carbonate (Saarchem) solution (75 g/l) was prepared in MilliQ™ water, heated at 30 ^{o}C until all the sodium carbonate was dissolved, and filter sterilized using a 0.22 µm Millex™ filter. Thereafter, it was stored at +4 ^{o}C until required.

7.2.4.1.2 Preparation of Folin-Ciocalteu reagent

A 1% (v/v) solution of the Folin-Ciocalteu (FC) reagent (Merck) was prepared in sterile MilliQ™ water and stored at +4 ^{o}C away from light.

7.2.4.1.3 Wavelength scan for the phenol and FC reagent

To determine the absorbance maxima of the phenol and FC reagent complex, gallic acid (100 mg/ml) was used and treated as outlined in Section 7.2.4.1.4 below, thereafter a wavelength scan was done on a Lambda 25 Perkin Elmer UV/VIS spectrometer, using UV WinLab Version 2.85.04, between 850 and 400 nm. An absorbance maxima was found at 638.74 nm (Appendix E; Figure E3). To test for interference, *O. sphaerocarpa* leaves (100 µg/ml) were treated the same way without adding the FC reagent. Upon taking absorbance measurements, a sharp peak at 672.09 nm (Appendix D3) was detected. This maxima is probably due to chlorophyll b which has an absorbance maxima between 650 and 680 nm (Iriyama *et al*., 1974), and therefore to minimize interference, absorbance measurement was shifted to 760 nm.

7.2.4.1.4 Preparation of gallic acid standard curve

Gallic acid (5 g; Sigma) was dissolved in 10 ml DMSO (Saarchem) to make a stock solution of 500 mg/ml. Dilutions ranging from 250 to 10 mg/ml were prepared in DMSO. FC reagent (500 µl) was added to 100 µl of the various extract dilutions, followed by 400 µl Na_2CO_3. This reaction mixture was allowed to stand in the dark at room temperature for 2 hours. Thereafter, absorbance was measured using a Philips PU8700 UV/Visible spectrophotometer at 760 nm. A standard curve was generated using Graphpad Prism® (version 4.0) (Figure 7.10), after three independent experiments were performed.

7.2.4.1.5 Determination of total phenols in extracts

A stock solution of 10 mg/ml of crude extract was prepared in DMSO. The reaction mixture consisted of 100 µl of the extract stock, 500 µl FC reagent and 400 µl Na_2CO_3. After standing in the dark at room temperature for 2 hours, the absorbance was measured at 760 nm. The concentration of total phenols in the extract was expressed as gallic acid equivalents (mgGAE/ g extract) using the gallic acid calibration curve (Djeridane *et al*., 2006; Wong *et al*., 2006; Kähkönen *et al*., 1999; Wojdylo *et al*., 2007; Mau *et al*., 2002; Bouayed *et al*., 2007). The formula below (Figure 7.11) was used to calculate the concentration of the phenolics.

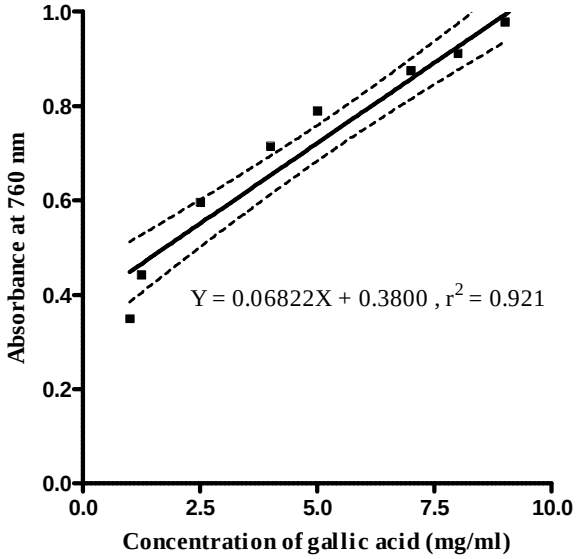


Figure 7.10: Gallic acid calibration curve, used to calculate the amount of phenolics in extract, plotted with a 95% confidence interval.

$$X = \frac{Y - 0.06822}{0.38}$$

where:

X = concentration of phenolics in gallic acid equivalents

Y = Absorbance of extract at 760 nm

Figure 7.11: Equation for the calculation of total phenolic content of extracts.

7.2.4.2 Flavonoid content

Flavonoid concentration in the extracts was estimated using a spectrophotometric method that is based on the formation of a flavonoid-aluminium complex with an absorbance maxima at 430 nm. Rutin, which is a flavonol glycoside comprised of the flavonol quercetin (Figure 7.12), was used to prepare a standard curve and the flavonoid content was calculated as milligram rutin equivalents per gram of extract (Quettier-Deleu *et al*., 2000).

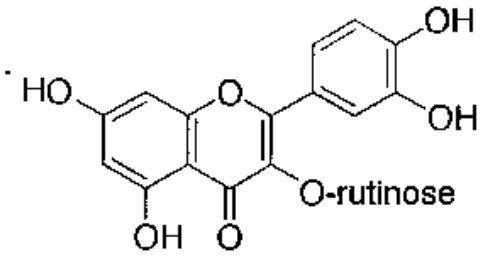


Figure 7.12: Chemical structure of rutin.

7.2.4.2.1 Preparation of aluminium sulphate

To prepare a 2% (w/v) solution, aluminium sulphate (2 g; British Drug Houses (BDH)) was dissolved first in 50 ml MilliQ™ water to promote dissolution. Thereafter, 50 ml HPLC grade methanol (Rochelle Chemicals) was added to make up the volume and the solution stored at +4 ^{o}C until required.

7.2.4.2.2 Wavelength scan for the rutin-aluminium complex

To determine the absorbance maxima of the rutin-aluminium complex, one concentration of rutin was used and treated as outlined in Section 7.2.4.2.3 below, thereafter a wavelength scan was done on a Perkin Elmer precisely Lambda 25 UV/VIS spectrometer, using UV WinLab Version 2.85.04, between 700 and 300 nm. An absorbance maxima was detected at 394.65 nm (Appendix E; Figure E4). The method was then adapted to a microtitre plate and absorbance measured at 405 nm as this was the only available filter in our lab closest to the wavelength maxima.

7.2.4.2.3 Preparation of rutin standard curve

Rutin (0.5 g; Sigma-Aldrich) was dissolved in 15 ml HPLC grade methanol, stirred until dissolved and stored in the dark at +4 ^{o}C. To prepare a working solution of 0.833 mg/ml, 20 µl of the 33.33 mg/ml stock was diluted with 780 µl methanol and subsequently serially diluted (1:1) to prepare 10 concentrations (417 to 0.8 µg/ml). The dilutions (100 µl) were plated out in triplicate wells in a 96-well microtitre plate, followed by 100 µl aluminium sulphate. The plate was incubated at room temperature in the dark for 15 minutes. Absorbance was measured at 405 nm on the Labsystems iEMS Reader MF using the Ascent

software (version 2.4). The experiment was done in triplicate and thereafter, absorbance was plotted against rutin concentration to obtain the standard curve (Figure 7.13).

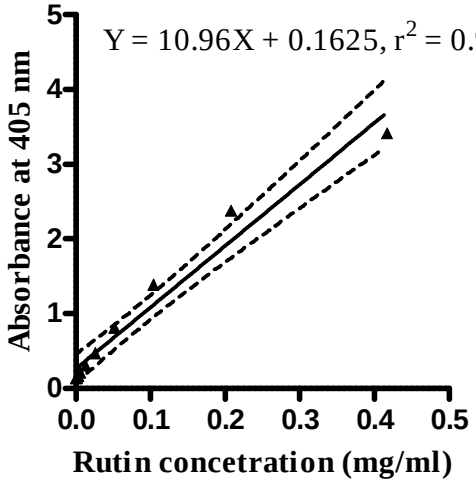


Figure 7.13: Rutin calibration curve used to calculate the concentration of flavonoids in the extracts.

7.2.4.2.4 Determination of flavonoid content in extracts

The plant extract (100 µl; dissolved in DMSO) at a concentration of 10 mg/ml was plated in triplicate wells on a 96-well microtitre plate. Aluminium sulphate (100 µl) was added to two of the wells. The third well acted as a colour control and only methanol was added. The plate was incubated at room temperature in the dark for 15 minutes. Thereafter absorbance was measured at 405 nm. Flavonoid content was calculate from the rutin standard curve and expressed as milligram rutin equivalents per gram of extract (mgRE/ g extract) using the equation in Figure 7.14.

$$X = \frac{Y - 0.1625}{10.96}$$

where:

X = concentration of flavonoids in rutin equivalent

Y = Absorbance of extract at 405 nm

Figure 7.14: Equation for the calculation of flavonoid content of extracts.

7.2.5 Data analysis

All the experiments in this section were done in triplicate and the results are presented as mean ± s.d.. ANOVA was used to compare extract activity with the controls using Graphpad Prism® (version 4.0). A p value of less than 0.05 ($p<0.05$) was considered significant. Linear regression was used to determine correlation between the different anti-oxidant activity assays and phenolic or flavonoid content with a 95% confidence interval.

7.3 Results

7.3.1 DPPH radical scavenging activity

Several of the extracts possessed the ability to scavenge the DPPH· radical (Table 7.1; Figure 7.15); while 39.4% of the plants did not possess (IC_{50}>100 µg/ml). *Dichrostachys cinerea* (leaves and fruit) and *Ficus glumosa* (bark) were the most potent DPPH· radical scavengers with IC_{50} values of 5.89 ± 0.39, 6.02 ± 0.79, 5.84 ± 1.53 µg/ml, respectively; which were comparable to ascorbic acid with an IC_{50} value of 5.61 ± 1.13 µg/ml ($p>0.05$). There was also no significant difference ($p>0.05$) between ascorbic acid and *Terminalia phanerophlebia* (leaves) and *Syzygium cordatum* (bark) (Table 7.1).

The results obtained from the HPTLC plates indicate that there are several compounds within each species contributing to the overall DPPH· radical scavenging activity of the plants (Figure 7.16A and 7.16B). Of the twenty plants assayed using the HPTLC method, five showed very poor DPPH· radical scavenging potential. These were *P. meyeri* (whole plant), *F. glumosa* (fruit), *T. emetica* (leaves) (Figure 7.16A, tracks 3, 7 and 9) and *Z. mucronata* (leaves) (Figure 7.16B, track 6); whilst *O. sphaerocarpa* (fruit) did not show any decolourisation of the DPPH· radical (Figure 7.16B, track 8). In contrast, the fruit and leaves of *D. cinerea* showed good decolourisation of the DPPH· radical using the HPTLC method, indicating the presence of many compounds with anti-oxidant activity within this plant (Figure 7.16A, track 4 and 5). These results are in agreement with the microtitre plate method which showed DPPH· radical scavenging of both the plants parts, with IC_{50} values comparable to ascorbic acid (Table 7.1).

7.3.2 Metal chelating activity

Thirty nine percent (39.4%) of the extracts exhibited metal chelating activity, with IC_{50} values greater than 100 µg/ml (Table 7.1; Figure 7.17). *Terminalia phanerophlebia* bark (IC_{50}: 22.72

Table 7.1: Anti-oxidant activity of selected Swazi traditional phytomedicines.

Scientific Name	Plant part	DPPH· radical scavenging (µg/ml) (IC_{50} ± s.d)	Metal chelation (µg/ml) (IC_{50} ± s.d)	Inhibition of lipid peroxidation % Inhibition at 100µg/ml ± s.d)
Berkheya setifera	flower	>100	>100	24.66 ± 4.38
	stem/root	>100	>100	59.45 ± 2.09
	leaves	>100	>100	2.54 ± 0.47
Breonadia salicina	leaves	25.86 ± 2.86	>100	58.97 ± 2.65
	bark	24.91 ± 3.14	>100	70.72 ± 1.39
Dichrostachys cinerea	fruit	6.02 ± 0.79	17.37 ± 0.77	95.03 ± 3.25
	leaves	5.89 ± 0.39	36.27 ± 3.01	60.68 ± 2.94
Ficus glumosa	fruit	70.09 ± 8.20	>100	55.44 ± 2.85
	leaves	46.76 ± 0.64	>100	65.62 ± 3.78
	bark	5.84 ± 1.53	39.02 ± 3.41	12.61 ± 1.88
Gardenia spatulifolia	fruit	>100	>100	58.24 ± 1.47
	leaves	21.57 ± 4.00	>100	77.55 ± 2.58
G. densa	whole plant	>100	>100	23.70 ± 3.80
Helichrysum acutatum	leaves	109.75 ± 9.50	31.68 ± 2.23	46.34 ± 3.04
	roots	>100	>100	51.67 ± 3.13
Leonotis intermedia	flower	>100	>100	31.68 ± 3.44
	stem	>100	>100	11.87 ± 1.75
Ozoroa sphaerocarpa	fruit	11.63 ± 0.79	40.59 ± 3.52	76.47 ± 2.66
	leaves	34.44 ± 4.08	58.72 ± 4.01	43.90 ± 1.09
	bark	11.23 ± 0.82	67.87 ± 4.08	80.10 ± 2.21
Priva meyeri	whole plant	70.04 ± 7.27	>100	84.37 ± 1.89
Ricinus communis	fruit	>100	>100	63.74 ± 4.39
	leaves	>100	32.80 ± 1.51	88.46 ± 3.25
	stem	>100	>100	16.98 ± 2.53
Syzygium cordatum	leaves	15.76 ± 2.37	>100	73.07 ± 3.90
	bark	9.54 ± 0.92	16.23 ± 0.99	82.33 ± 2.21
Terminalia phanerophlebia	leaves	7.09 ± 0.78	14.43 ± 1.32	91.63 ± 1.16
	bark	12.37 ± 3.03	22.72 ± 1.33	84.27 ± 0.49
Trichilia emetica	leaves	70.59 ± 12.68	44.21 ± 4.25	59.05 ± 1.83
	bark	15.16 ± 1.75	>100	59.73 ± 2.59
Ziziphus mucronata	fruit	43.54 ± 1.24	>100	26.43 ± 2.04
	leaves	>100	>100	28.66 ± 1.18
	bark	15.27 ± 2.22	55.67 ± 1.71	38.73 ± 2.36
Controls (a: Ascorbic acid; b: EDTA; c: Trolox™)		5.61 ± 1.13[a]	20.86 ± 2.53[b]	93.94 ± 3.17[a], 48.49 ± 3.58[c]

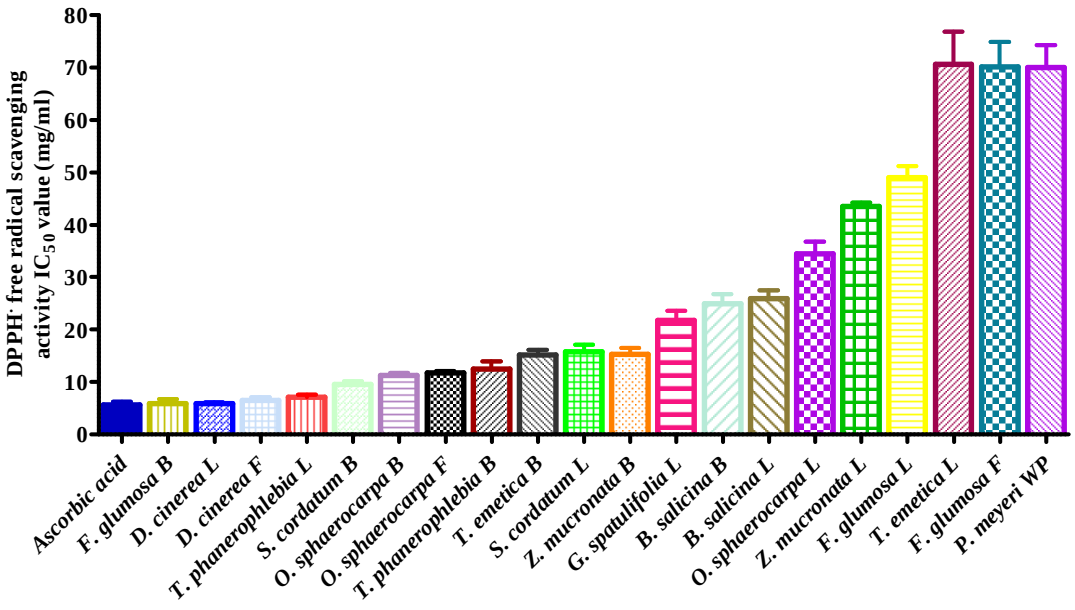
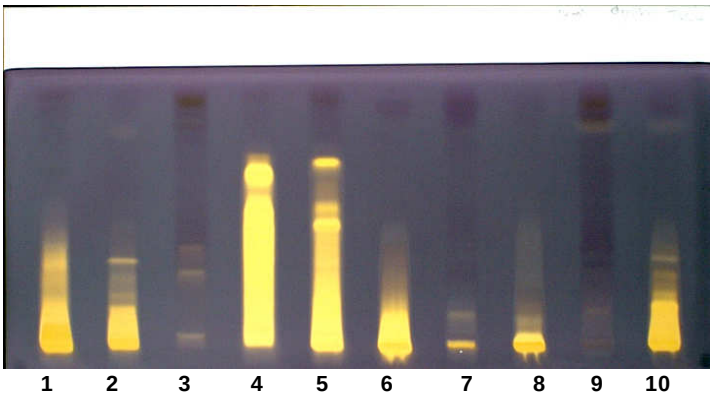
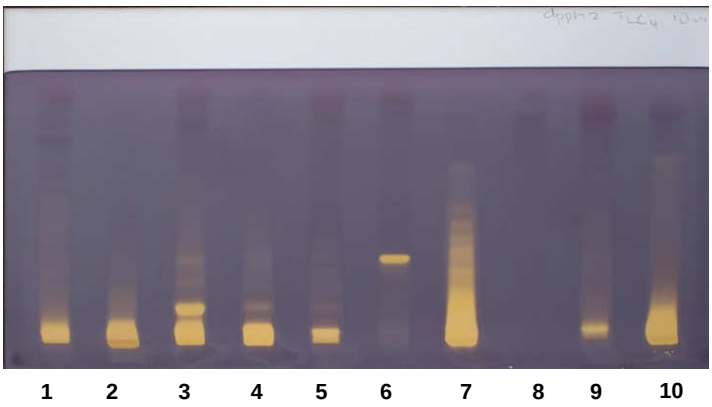


Figure 7.15: DPPH· free radical scavenging activity of the most active extracts, where: L = leaves, B = bark, F = fruit/flower, WP = whole plant.



Legend:
1 – *T. phanerophlebia* leaves
2 – *T. phanerophlebia* bark
3 – *P. meyeri* whole plant
4 – *D. cinerea* fruit
5 – *D. cinerea* leaves
6 – *F. glumosa* bark
7 – *F. glumosa* fruit
8 – *F. glumosa* leaves
9 – *T. emetica* leaves
10 – *T. emetica* bark

Figure 7.16A: HPTLC plate (A) dipped in DPPH· to display compounds with free radical scavenging potential.



Legend:
1 – *S. cordatum* leaves
2 – *S. cordatum* bark
3 – *B. salicina* leaves
4 – *B. salicina* bark
5 – *G. spatulifolia* leaves
6 – *Z. mucronata* leaves
7 – *Z. mucronata* bark
8 – *O. sphaerocarpa* fruit
9 – *O. sphaerocarpa* leaves
10 – *O. sphaerocarpa* bark

Figure 7.16B: HPTLC plate dipped in DPPH· to display compounds with free radical scavenging potential.

± 1.33 µg/ml), *D. cinerea* fruit (IC_{50}: 17.37 ± 0.77 µg/ml) and *S. cordatum* bark (IC_{50}: 16.23 ± 0.99 µg/ml) had metal chelating activity that was significantly ($p>0.05$) comparable to EDTA (IC_{50}: 20.86 ± 2.53 µg/ml). The leaves of *T. phanerophlebia* (IC_{50}: 14.43 ± 1.32 µg/ml) possessed a significantly ($p< 0.05$) higher metal chelating activity potential. There was no correlation between metal chelating activity of the extracts and DPPH· free radical scavenging activity ($r^2 = 0.23$), however, it was noted that extracts which did not possess DPPH· free radical scavenging activity (IC_{50} > 100 µg/ml) also displayed poor metal chelating activity (IC_{50} > 100 µg/ml).

7.3.3 Inhibition of lipid peroxidation

Inhibition of lipid peroxidation was determined by the linoleic acid system. Twenty one out of the thirty three extracts (64%) exhibited greater than 50% inhibition at a concentration of 100 µg/ml (Table 7.1). Ascorbic acid and Trolox™ were used as positive controls and at 100 µg/ml had 93.94 ± 3.17% and 48.49 ± 3.58% inhibition, respectively. *Dichrostachys cinerea* (fruit), *T. phanerophlebia* (leaves) and *R. communis* (leaves) had inhibitory properties comparable to ascorbic acid; 95.03 ± 3.25%, 91.63 ± 1.12% and 88.46 ± 3.25%, respectively ($p>0.05$). There was, however, no correlation between either DPPH· radical scavenging or metal chelating activity with lipid peroxidation inhibition at a 95% confidence interval, $r^2 = 0.24$ and 0.19, respectively. The leaves of *B. setifera* (2.54 ± 0.47%), stem of *L. intermedia* (11.87 ± 1.75%) and bark of *F. glumosa* (12.61 ± 1.88%) showed the least inhibitory activity in the linoleic

acid system. *Berkheya setifera* and *L. intermedia* did not show any anti-oxidant activity using either the DPPH· radical scavenging or metal chelating methods (Table 7.1).

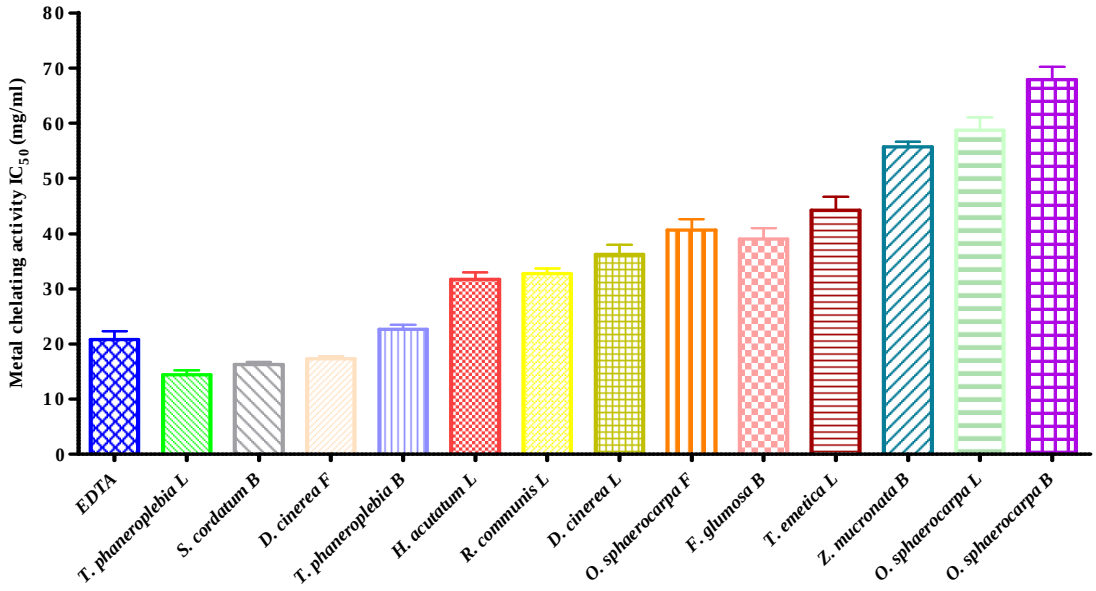


Figure 3.17: Metal chelating activity of the most active extracts, where: L = leaves, B = bark, F = fruit/flower.

7.3.4 Phenolic and flavonoid content

Phenolic content was estimated as gallic acid equivalents per gram of plant extract and the results are shown in Table 7.2. The leaves of *S. cordatum* and fruit of *Z. mucronata* had the highest content of phenolics (806.10 ± 42.28 and 536.87 ± 36.76 mg GAE/g extract), respectively. *Ricinus communis* (leaves), *B. setifera* (leaves) and *T. emetica* (leaves) also had high phenolic content (469.00 ± 55.72 to 329.00 ± 5.92 mg GAE/g extract). Only seven of the 33 extracts had no appreciable phenolic content (<0.01 mg GAE/g extract).

The flavonoid content as measured in rutin equivalents/g extract was much less than the total phenolic content found in each extract. *Terminalia phanerophlebia* (bark), *O. sphaerocarpa* (fruit), *S. cordautm* (bark) and *T. emetica* (bark) had the highest content of flavonoids (33.33 ± 4.43; 31.13 ± 1.10; 27.13 ± 2.39; 26.40 ± 0.72 mg RE/g extract, respectively).

Table 7.2: Phenolic and flavonoid content of selected Swazi traditional phytomedicines.

Scientific Name	Plant part	Phenolic content (mg GAE/g extract ± s.d)	Flavonoid content (mg RE/g extract ± s.d)
Berkheya setifera	flower	243.00 ± 3.03	13.00 ± 0.60
	stem/root	443.93 ± 44.71	<0.01
	leaves	260.73 ± 6.41	<0.01
Breonadia salicina	leaves	142.40 ± 4.00	12.20 ± 1.97
	bark	<0.01	3.40 ± 0.20
Dichrostachys cinerea	fruit	51.90 ± 3.07	2.10 ± 0.27
	leaves	<0.01	6.27 ± 0.61
Ficus glumosa	fruit	45.90 ± 0.78	14.07 ± 1.62
	leaves	<0.01	5.47 ± 0.12
	bark	<0.01	0.78 ± 0.03
Gardenia spatulifolia	fruit	110.60± 1.06	12.80 ± 1.40
	leaves	228.30 ± 2.43	2.53 ± 0.12
G. densa	whole plant	150.50 ± 0.99	<0.01
Helichrysum acutatum	leaves	182.80 ± 4.65	0.50 ± 0.10
	roots	<0.01	3.50 ± 0.10
Leonotis intermedia	flower	129.00 ± 1.57	<0.01
	stem	179.00 ± 3.5	4.13 ± 0.23
Ozoroa sphaerocarpa	fruit	<0.01	31.13 ± 1.10
	leaves	34.67 ± 2.25	1.30 ± 0.10
	bark	<0.01	<0.01
Priva meyeri	whole plant	295.43 ± 32.60	7.33 ± 1.16
Ricinus communis	fruit	263.37 ± 1.12	<0.01
	leaves	469.00 ± 55.72	4.07 ± 0.23
	stem	197.00 ± 0.44	<0.01
Syzygium cordatum	leaves	806.10 ± 42.28	4.10 ± 0.78
	bark	33.70 ± 1.13	27.13 ± 2.39
Terminalia phanerophlebia	leaves	184.70 ± 2.19	5.20 ± 0.20
	bark	174.70 ± 11.73	33.33 ± 4.43
Trichilia emetica	leaves	329.00 ± 5.92	<0.01
	bark	17.00 ± 1.89	26.40 ± 0.72
Ziziphus mucronata	fruit	536.87 ± 36.76	<0.01
	leaves	218.00 ± 5.76	3.40 ± 0.20
	bark	46.27 ± 5.61	<0.01

There was no correlation between anti-oxidant activity and phenolic or flavonoid content, with r^2 values of 0.02 and 0.13 for DPPH$^{\cdot}$ activity against phenolic and flavonoid content, respectively and r^2 values of 0.05 and 0.17 for metal chelating activity against phenolic and flavonoid content, respectively.

7.4 Discussion

Anti-oxidants have, on numerous occasions, been documented to possess many beneficial effects to mankind, such as in the prevention/treatment of cancer, rheumatoid arthritis, diabetes and hypertension. Many herbal drugs which have free radical scavenging potential have gained importance in treating chronic diseases which are exacerbated by free radicals (Farombi *et al*., 2003). Natural anti-oxidants such as vitamins (C and E), polyphenols or flavones play a significant role in scavenging free radicals. Herbal preparations containing free radical scavenging activity can play a role as anti-oxidants and help minimize their deleterious effects.

In the present study, the anti-oxidant activity of 33 dichloromethane/methanol extracts from 15 plant species used as traditional medicine in Swaziland was assessed. Three anti-oxidant assays were used to determine if the extracts had any activity and also assess their possible mode of action. DPPH$^{\cdot}$ free radical scavenging was used as it is a widely used assay to determine free radical scavenging activity (Lourens *et al*., 2004; Miliauskas *et.al*., 2004; Gülçin *et al*., 2005; Zhan *et al*., 2006; Kamatou *et al*., 2007). In this assay, the stable DPPH radical is reduced to a yellow coloured diphenyl-picrylhydrazine by anti-oxidants. A number of the extracts showed significant ability to scavenge the DPPH$^{\cdot}$ radical, such as the fruit and leaves of *D. cinerea*, the bark of *F. glumosa* and the bark of *S. cordatum* (Table 7.1).

The iron chelating activity was determined using the iron-ferrozine complex assay. Iron is an important catalyst in the Fenton reaction (Figure 7.2) which generates the hydroxyl radical (Nordberg and Arnèr, 2001). Therefore, chelation of this metal ion and thus preventing its involvement in the Fenton reaction may, be important in reducing the production of the hydroxyl radical involved in lipid peroxidation and DNA or protein damage.

It would be expected that upon the chelation of these metal ions, lipid peroxidation would also be reduced as the precursor to this reaction has been chelated. The results of this study are consistent with this, as the leaves and bark of *T. phanerophlebia*, fruit of *D. cinerea* and bark of *S. cordatum*, all showed good metal chelation and lipid peroxidation inhibition activity. However, some other extracts showed good lipid peroxidation inhibition but had no metal chelating activity, such as the leaves of *S. cordatum* and *G. spatulifolia*, bark of *B. salicina* and *P. meyeri* whole plant (Figure 7.15; Table 7.1). This indicates that the Fenton reaction is not the only source of hydroxyl radicals. Secondly, alkoxyl radicals, produced by other mechanisms, can also initiate lipid peroxidation (Kohen and Nyska, 2002).

The four extracts (leaves and bark of *T. phanerophlebia*, fruit of *D. cinerea* and bark of *S. cordatum)* also had good DPPH$^{\cdot}$ radical scavenging activity (Table 7.1), which might suggest that the radical scavenging and metal chelation work hand-in-hand to produce the overall effect on lipid peroxidation. Extracts which possessed only DPPH$^{\cdot}$ free radical scavenging or metal chelating activity, did not show appreciable lipid peroxidation inhibition, which might be because the radicals scavenged are not responsible for initiating lipid peroxidation (Kohen and Nyska, 2002).

The anti-oxidant activity can, in part, be attributed to the phenolic and flavonoid content of these extracts. *Syzygium cordatum* (leaves) which had good anti-oxidant activity also had a high content of phenolics (Table 7.1 and 7.2). The other three extracts (*T. phanerophlebia* leaves and bark and *D. cinerea* fruit) had either a high phenolic or flavonoid content (Table 7.2). Phenolic compounds of the plant kingdom have been reported to have multiple biological effects including anti-oxidant activity (Hou *et al*., 2003). It has been suggested that the phenolic compounds may contribute directly to anti-oxidant action and also play a role in stabilizing lipid peroxidation (Yen *et al*., 1993; Duh *et al*., 1999). In fact, some studies have shown a positive linear correlation between anti-oxidant activity and phenolic content of a plant. Djeridane *et al*. (2006) tested the anti-oxidant activity of a number of Algerian plants using the ABTS assay, which uses the same principle as the DPPH$^{\cdot}$ method, and found a correlation with the phenolic content, measured using the FC-reagent with gallic acid as a standard (r^2 = 0.79). This is not a desired value when dealing with linear regression, but it does indicate a correlation between the anti-oxidant activity and phenolic content of the plants

in thier study. Similarly, Wojdylo *et al.* (2007) reported correlation between phenolic content measured using an HPLC method and anti-oxidant activity using the DPPH, ABTS and ferric reducing/anti-oxidant power assays (r^2 = 0.84, 0.93, 0.91), respectively. However, in the present study, a linear correlation was not found between the phenolic content and anti-oxidant activity as measured with the DPPH assay and metal chelating activity assay (r^2 = 0.02 and 0.05), respectively.

The anti-oxidant activity of *S. cordatum* may also be attributed to proanthocyanidins and pentacyclic triterpenoids, which are found in the bark and wood and have been reported to possess potent anti-oxidant activity (Škerget *et al.*, 2005). The stained HPTLC plates also showed some anti-oxidant activity in the plant though it was not very high (Figure 7.16B, track 1 and 2). The high content of gallic acid in the leaves (highest of all extracts) is in agreement with other studies which have shown that it contains gallic acid, ellagic acid and various gallic acid derivatives (van Wyk, 2000). Wojdylo *et al*. (2007) has reported the DPPH radical scavenging potential and total phenolic content of the fruit of *S. aromaticum* to be 884 ± 9.04 μM Trolox™/100g dry weight of extract and 8.96 ± 0.34 mg GAE/100g dry weight, respectively; which are quite high.

Terminalia species are known to contain several pentacyclic triterpenoids, tannins, triterpene saponins and ellagic acid (van Wyk, 2000; Neuwinger, 1996), which may be responsible for the anti-oxidant activity found in *T. phanerophlebia*. The difference between the activity of the bark and leaves was also seen on the HPTLC plates where the leaves showed more discolouration compared to the bark (Figure 7.16A, tracks 1 and 2). The anti-oxidant activity of *T. catappa* leaves using the DPPH assay, ferrous metal chelating assay and the lipid peroxidation assay have been reported by Chyau *et al.* (2002), where the %DPPH· decolourisation was between 92.5 and 95.7% at 100 μg/ml, similar to what was obtained in this study (95.13 ± 1.15%) before determining the IC_{50} value.

Dichrostachys cinerea (leaves and fruit) and *F. glumosa* (bark) had activity comparable to the potent anti-oxidant ascorbic acid (Figure 7.15; Table 7.1). The activity was also evident in the HPTLC method where a number of compounds possess the ability to decolourise the DPPH· radical (Figure 7.16A, track 4 and 5). These compounds may interact synergistically to

generate the overall activity observed in the microtitre plate method. *Dichrostachys cinerea* has been shown to contain hentriacontanol, β-amyrin and sitosterol, which may be responsible for the anti-oxidant activity it has exhibited in this study (Joshi and Sharma, 1974). Furthermore, Rao *et al*. (2003) isolated mesquitol and epicatechin from the *D. cinerea* and further reported potent DPPH and ABTS radical scavenging activity of mesquitol, whilst epicatechin was reported to scavenge the $DPPH^{\cdot}$ radical. Both the fruit and leaves of *D. cinerea* did not possess significant amounts of phenolics and flavonoids (Table 7.2).

Ficus species have been previously reported to possess anti-oxidant activity. This is confirmed by the current results which displayed $DPPH^{\cdot}$ radical scavenging activity of the bark (IC_{50}: 5.84 ± 1.53 μg/ml) which was not significantly different from ascorbic acid (IC_{50}: 5.61 ± 1.13 μg/ml) (Table 7.1). The HPTLC plates also displayed the greater activity of the bark in comparison with the leaves or fruit (Figure 7.16A, track 6). These results were similar to those obtained in a study by Ao *et al*. (2008), whereby an EC_{50} of 7.9 ± 0.1 μg/ml was obtained for the bark methanolic extract of *F. microcarpa* using the $DPPH^{\cdot}$ radical scavenging assay. The fruit also had a high activity (IC_{50}: 7.3 ± 0.0 μg/ml), which is about tenfold less than what was obtained in this study for the fruit of *F. glumosa* (IC_{50} value of 70.9 ± 8.2 μg/ml). Leong *et al*. (2008) reported the presence of a flavonoid glycoside with $DPPH^{\cdot}$ radical scavenging activity in the leaves of *F. pumila* L. Contrasting results are reported by Annan and Houghton (2008) for the $DPPH^{\cdot}$ radical scavenging activity of *F. asperifolia*; where an IC_{50} value of 237 μg/ml was obtained, which is 40-fold greater than what was obtained in this study. There was also a huge difference in the phenolic/flavonoid content of the extracts, the results in this study reports low concentrations of phenolics (<0.01 mg GAE/g extract) and flavonoids in all the parts (Table 7.2), whilst Ao *et al*. (2008) reported a high content of phenolics in the bark (237 ± 0.6 mg GAE/g extract), which correlates to the high free radical scavenging activity reported.

All parts of *Leonotis intermedia* displayed insignificant anti-oxidant activity, with both DPPH radical scavenging and metal chelation, having IC_{50} values greater than 100 μg/ml (Figure 7.15; Table 7.1). A study by David *et al*. (2007) on *L. nepetifolia* (aerial part methanolic extract) reported an IC_{50} value of 6.5 mg/ml for the $DPPH^{\cdot}$ radical scavenging activity. The results are in agreement with our observation of a lack of $DPPH^{\cdot}$ radical scavenging potential

in this plant, even though the phenolic content was approximately 150 mg GAE/g extract (Table 7.2).

The leaves of *G. spatulifolia* displayed some anti-oxidant activity, with an IC_{50} value of 21.57 ± 4.0 µg/ml, which was observed in the HPTLC plates (Figure 7.16B, track 5). A previous study by Chen *et al*. (2008) demonstrated anti-oxidant activity of gardecin and crocins isolated from the fruit of *Gardenia jasminoides*. *G. spatulifolia* is reportedly used in traditional medicine for the treatment of diabetes mellitus (Section 1.5.7); where low levels of plasma anti-oxidants have been implicated in the risk of developing diabetes mellitus (McCune and Johns (2002). The phenolic content of 228 mg GAE/g extract and to a lesser extent the flavonoids of *G. spatulifolia* could help to boost the plasma concentration of anti-oxidants to benefit diabetic patients.

O. sphaerocarpa possesses a high degree of anti-oxidant activity, with the bark and fruit displaying potent DPPH radical scavenging activity and inhibition of lipid peroxidation (Figure 7.15; Table 7.1). Contrary to this, the HPTLC plates did not show high activity by the fruit (Figure 7.16B, track 8). The fruit also displayed a high content of flavonoids compared to the other parts. A literature search did not reveal any work done on the anti-oxidant activity of this species or genus, therefore, to the best of our knowledge, this is the first documentation of anti-oxidant activity in the plant and genus.

Although *R. communis* is a widely used plant, no literature on its anti-oxidant activity could be found. The present study displays some metal chelating activity and lipid peroxidation inhibition of the leaves (Table 7.1). In agreement with the anti-oxidant results, the leaves also show a high content of phenolics (Table 7.2).

H. acutatum did not display any anti-oxidant activity even though it had a high content of phenolics (182.8 ± 4.65 mg GAE/g extract) (Table 7.2). Lourens *et al*. (2004) reports contrasting results for four species of *Helichrysum*, with $DPPH^{\cdot}$ radical scavenging activity for *H. dasyanthum*, *H, excisum*, *H. felinum* and *H. petiolare* having IC_{50} values of 12.33 ± 2.38, 13.67 ± 0.23, 20.71 ± 5.42 and 28.7 ± 4.61 µg/ml, respectively.

Overall, *T. emetica* (leaves and bark) did not show appreciable anti-oxidant activity with only the bark possessing some $DPPH^{\cdot}$ radical scavenging potential, with an IC_{50} value of 15.16 ± 1.75 μg/ml (Table 7.1). This is seen in the HPTLC plates, where there was a high decolourisation in the bark extract (Figure 7.16A, track 10). The leaves had a high content of phenolics (329 ± 5.92 mg GAE/g extract) and the bark had a high content of flavonoids (26.4 ± 0.72 mg RE/g extract) (Table 7.2). Germano *et al.* (2006) reported the presence of phenolic acids in the root of *T. emetica*, but these exist in bound forms and can hardly be detected as free compounds.

Z. mucronata did not possess anti-oxidant activity, except for the bark which displayed some degree of $DPPH^{\cdot}$ radical scavenging activity (Figure 7.15). Only one compound in the leaves was responsible for the $DPPH^{\cdot}$ radical scavenging, otherwise, the bark had a high concentration of compounds responsible for $DPPH^{\cdot}$ radical scavenging (Figure 7.16B, track 7). All the parts had a high content of phenolics (Table 7.2).

G. densa, *B. setifera* and *P. meyeri* did not have anti-oxidant activity, while the leaves and bark of *B. salicina* only possessed DPPH radical scavenging activity, with IC_{50} values of 25.86 ± 2.86 and 24.91 ± 3.14 μg/ml, respectively (Figure 7.14; Table 7.1). All four plants possessed phenolic and flavonoid content with the stem/root extract of *B. setifera* exhibiting the highest phenolic content (443. 93 ± 44.71 mg GAE/g extract) (Table 7.2). This work has for the first time reported on the anti-oxidant activity of these four plants.

In conclusion, the fruit of *D. cinera*, bark of *S. cordatum* and leaves and bark of *T. phanerophlebia* were found to be good sources of anti-oxidants as they displayed good DPPH free radical scavenging, metal chelating and lipid peroxidation inhibition activity. They also have a relatively high content of phenolics and flavonoids which may be responsible for the anti-oxidant activity. These plants may be of use in the pharmaceutical industry as sources of natural anti-oxidants.

CHAPTER 8: ANTI-DIARRHOEAL EFFECTS OF *Breonadia salicina*, *Syzygium cordatum* AND *Ozoroa sphaerocarpa*

8.1 Introduction

8.1.1 Aetiology of diarrhoea

Diarrhoeal disease is among the six biggest infectious killers accounting for 1.9 million deaths in developing countries, and about 4-5 million deaths worldwide. It is the third leading cause of death in children in developing countries, accounting for 15.2% of all childhood deaths and is associated with inadequate water supply and sanitation (Boutayeb, 2006; Mukherjee *et al*., 1998). According to the World Health Organization, diarrhoea is three or more loose or watery stools passed in a period of 24 hours and can be classified as acute or chronic (Palombo, 2006). Pharmacologically, diarrhoea is defined as an increased fluidity and volume of faeces or an increased frequency of defecation. It is essentially caused by an increased colonic volume which results in distension of the colon and triggering of the defecation reflux. The increased colonic volume may be due to:

- irritation as a result of bacterial, viral or parasitic infections, laxatives or some gastrointestinal tumours or medication,
- increased osmotic pressure due to impaired digestion or absorption or
- decreased water absorption resulting from malabsorptive disorders (Bunnett and Lingappa, 2006).

Another form of diarrhoea is psychogenic diarrhoea which normally develops as a result of excessive stimulation of the parasympathetic nervous system leading to increased motility and secretion of mucus in the distal colon (Coker *et al*., 1992).

For the purpose of this study, diarrhoea resulting from bacterial infections is the focal point. The human body is colonized by various micro-organisms which are part of the normal flora. Among these are the *Escherichia coli*, other enterobacteriaceae, enterococci and yeasts which colonize the upper bowel. The lower bowel is also colonized by the same pathogens, but in addition has actinomycetes, *Bacteriodes* species, *Clostridium* species, bifidobacteria and eubacteria. The micro-flora are capable of causing disease under certain conditions, such as

host factors, which may include previous immunity, nutrition and underlying disease and environmental factors such as temperature, dust, humidity and use of antibiotics and pesticides (Bannister *et al*., 2000). Worldwide, the majority of infectious diarrhoea is caused by rotavirus infections, particularly among young children. Other causative agents include *Escherichia coli*, *Salmonella* spp, *Shigella* spp, *Campylobacter*, and *Vibrio cholerae* as well as *Cryptosporidium, Ascaris lumbridoides* and *Giardia* (Bannister *et al*., 2000; Palombo, 2006; Marcos and DuPont, 2007). Among these causative agents, it is reported that diarrhoeagenic *E. coli* is among the most important bacterial enteric pathogens, particularly in developing countries, accounting for under half of the cases of endemic paediatric diarrhoea and approximately half the cases of diarrhoea among international travellers to these areas (Marcos and DuPont, 2007; Moreno *et al*., 2008; Prère *et al*., 2006).

DEC is classified into six strains or groups; enterotoxigenic *E. coli*, enteroinvasive *E. coli*, enteroaggregative *E. coli*, diffusely adhering *E. coli*, entero haemorrhagic *E. coli* or most commonly referred to as shiga-toxin *E. coli* and enteropathogenic *E. coli* (Moreno *et al*., 2008; Bannister *et al*., 2000), with the latter two playing an important role in the aetiology of diarrhoea (Prère *et al*., 2006). In a study conducted in France, diarrhoeagenic bacteria (enteropathogenic and entero haemorrhagic *E. coli*) were the most prevalent enteric pathogens identified in the stools of 55 patients (Prère *et al*., 2006). It has also been reported in developing countries that enteropathogenic *E. coli* is the leading cause of childhood diarrhoea (Nataro and Kaper, 1998; Moreno *et al*., 2008). It is with these observations in mind that *E. coli* was chosen as a test agent for anti-diarrhoeal activity of *Breonadia salicina*, *Syzygium cordatum* and *Ozoroa sphaerocarpa*.

8.1.2 Treatment of acute diarrhoea

In modern medicine, there are four approaches to the treatment of acute diarrhoea:

- maintenance of the fluid and electrolyte balance with the use of sodium chloride and glucose solutions (oral rehydration therapy),
- use of anti-infective agents such as cotrimoxazole and metronidazole;
- use of non-antimicrobial anti-diarrhoeal agents such as loperamide, diphenoxylate, codeine and fluid adsorbents (kaolin, chalk, pectin, methylcellulose, bran, ispaghula husk);

- and most recently the use of probiotics or microbial components which have a value in the treatment of rotavirus infections, post antibiotic diarrhoea (Marcos and DuPont, 2007).

Traditional healers probably use the same approach in the treatment of diarrhoea, by using plants with antimicrobial activity or those that decrease gastric motility. There are many reports about the use of plants in the treatment of diarrhoea. Some of these plants include the leaves of *Lippia javanica*; bark and leaves of *Syzygium cordatum*; leaves from *Annona senegalensis*; root, tubers and fruit of *Rhoicissus tridentate* (Samie *et al*., 2005); leaves of *Psidium guajava;* bark of *Syzygium guineense* (Amusan *et al*., 2002); the bark, fruits and leaves of *Acacia nilotica*; leaves and bark of *Acanthospermum hispidum*; bark of *Gmelina arborea*; bark of *Parkia biglobosa* and *Vitex doniana* (Agunu *et al*., 2005). Mukherjee *et al*. (1998) reported the anti-diarrhoeal activity of some plants used in West Bengal, India. These plants were found to effect anti-diarrhoeal activity by inhibiting gastrointestinal motility and PGE_2-induced enteropooling and included, *Ficus bengalensis* Linn., *Eugenia jambolana* Lam., *Ficus racemosa* Linn. and *Leucas lavandulaefolia* Rees.

8.1.3 Combination therapy in modern medicine

The use of combination therapy in clinical practice is very common, especially for the treatment of infectious and malignant diseases. This is employed for the therapeutic advantages they may provide over single agents. Combination therapy is also employed to increase the spectrum of antimicrobial activity, to prevent treatment failure when antimicrobial resistance is suspected, prevent the development of resistance, to decrease dose-related toxicity by using less of a toxic antimicrobial agent and more of the non-toxic one and to obtain enhanced antimicrobial killing or inhibition. The drugs used in combination may have different mechanisms of action as well as affect different sites of the body, but the overall effect of the combination may either exceed the expected effect (synergism) or nullify each other's biological effects resulting in a reduced effect (antagonism). An additive interaction is defined as the effect where the combined action is equivalent to the sum of the actions of each drug when used alone (Berenbaum, 1989; Boucher and Tam, 2006; Brooks *et al*., 2007). There are three major mechanisms of antimicrobial synergy that have been established. These are:

- blockade of sequential steps in a metabolic sequence, such as the action of trimethoprim-sulfamethoxazole which blocks two sequential steps in the folic acid pathway;
- inhibition of enzymatic inactivation, such as the use of a β-lactam, such as amoxicillin together with a β-lactamase inhibitor (e.g. clavulanic acid) to prevent the inactivation of a β-lactam by β-lactamases, in the combination amoxiclav or;
- enhancing the uptake of an antimicrobial agent, such as the use of penicillins and other cell water active agents to increase the uptake of aminoglycosides such as gentamicin or streptomycin, resulting in synergism (Brooks *et al*., 2007; Lampiris and Maddix, 2007).

Antimicrobial antagonism on the other hand, rarely produces clinically relevant or desirable effects.

8.1.4 Combination therapy in traditional medicine

Traditional medicine is extensively used in Africa with 3 689 taxa (15.4%) of the 24 000 species used ethnomedicinally in the southern African region (Eloff and McGaw, 2006). In South Africa alone, there are an estimated 200 000 indigenous traditional healers who use various plant and plant combinations to prepare their remedies (van Wyk *et al*., 1997). In traditional medicine, different plants are combined for the treatment of a disease. The plants used are often not related in any way or may be used singularly for the treatment of that ailment. However, they have been found to achieve a better therapeutic effect when in combination (Personal communication with Mr. PP Ndlovu, traditional healer). A number of combination treatments are prepared by traditional healers, such as the use of *Terminalia sericea* root powder in combination with *Vigna unguiculata* seeds for the treatment of bilharzia (Neuwinger, 1996). A herbal mixture available already packaged is Imbiza ephuzwato™, which has up to 21 plant species in combination, and is used by the Zulu people of South Africa for the treatment of various ailments (Ndhlala *et al*., 2008). Of the 190 traditional medicine recipes recorded during the Organization of African Unity/Scientific, Technical and Research Commission (OAU/STRC) ethnobotanical survey of Swaziland, 52 (27.4%) were recipes prepared with two or more plant combinations (Adeniji *et al.*, 2000).

The bark of *O. sphaerocarpa, B. salicina* and *S. cordatum* are traditionally used in combination in the treatment of diarrhoea (Figure 8.1). In the preparation of the traditional remedy, these plants are used in a 1:1:1 ratio (Personal communication with Mr. PP Ndlovu, traditional healer). With the aim to determine the kind of antimicrobial interaction that exists between these three plants, a combination study was designed using all three plants against a common diarrhoea pathogen, *E. coli*.

(a)  (b)  (c) 

Figure 8.1: The bark of (a) *O. sphaerocarpa*, (b) *S. cordatum* and (c) *B. salicina*, which are used in combination for diarrhoea.

8.1.5 Implications of combination therapy on toxicity

Although it may be beneficial to combine drugs to increase effectiveness or avoid treatment failure in antimicrobial therapy, the implications of the combination on toxicity need to be explored. Two drugs may give the desired synergistic effect when used in combination for the treatment of a microbial infection, but prove to be toxic to the human/host's cells. As an example, the combined use of two nephrotoxic drugs can result in increased toxicity (synergistic effect) even though the individual doses may not be sufficient to produce such toxicity (Horn, 2007). Such toxicity is not limited only to clinical drugs; herbal preparations can also interact and result in potentiated toxicity. Thus there is a need to investigate the toxic effects or possible interactions that may result when combining plants in the treatment of disease.

8.2 Materials and Methods

8.2.1 Antimicrobial combinations

To scientifically verify the combined effect of the bark of *O. sphaerocarpa, B. salicina* and *S. cordatum,* against a common diarrhoea-causing pathogen, *Escherichia coli* (ATCC 25922), a combination study was designed. The antibacterial activity of all three possible plant combinations in various ratios, as well as the triple combination that is traditionally used, was tested to determine the type of pharmacological interaction between these plant combinations.

8.2.1.1 Screening for combined activity

This constituted the initial screening for antimicrobial activity when the extracts were combined in different ratios. A stock concentration of 85.3 mg/ml extract (Section 2.3) in acetone was prepared for each of *B. salicina*, *O. sphaerocarpa* and *S. cordatum*, and then mixed in separate eppendorf tubes according to Table 8.1. Each eppendorf tube was treated as a separate concentration mixture.

In order to maintain the dilution factor, water (100 µl) was added in place of the extract where that particular extract was not to be added (denoted by zero in the table). The resulting mixture comprising of 28.43 mg/ml of each extract was then plated (100 µl) in a 96-well microtitre plate in duplicate and serially diluted in a 1:1 ratio to produce a final concentration range of 7.13 mg/ml to 0.06 mg/ml after addition of the microbial culture. Antimicrobial activity was then determined as previously described using the MIC assay (Section 4.2.2.4). Three independent experiments were performed and the results averaged. Ciprofloxacin (0.01 mg/ml) in acetone was used as a positive control.

Table 8.1: Ratios and volumes used to combine the three plants in the initial screen for antimicrobial activity.

Ratio	***O. sphaerocarpa***	***S. cordatum***	***B. salicina***
1:0:0	100 µl	0	0
0:0:1	0	0	100 µl
0:1:0	0	100 µl	0
1:1:1	100 µl	100 µl	100 µl
1:1:0	100 µl	100 µl	0
1:0:1	100 µl	0	100 µl
0:1:1	0	100 µl	100 µl

8.2.1.2 Two plant combination study

The plant extracts were prepared at a concentration of 64 mg/ml in acetone. In separate eppendorf tubes, the different ratios of extracts were prepared (Table 8.2.), such that the required volume of 100 µl was prepared for each well. The three possible combinations were used in this study: *S. cordatum* with *O. sphaerocarpa*, *B. salicina* with *O. sphaerocarpa* and *S. cordatum* with *B. salicina*. The extract combinations were plated in duplicate in a 96-well microtitre plate and the concentrations ranged from 16.00 mg/ml to 0.013 mg/ml depending on the ratio used (Table F1). Seven serial 1:1 dilutions were prepared to determine the MIC of each combination (Section 4.2.2.4). The experiment was repeated three times and the results averaged. Ciprofloxacin (0.01 mg/ml) in acetone was used as a positive control.

Table 8.2: Ratios of plant extracts used to determine the two plant combination MIC.

Plant	**Ratio and volumes (100 µl) of Plant A : Plant B**										
A.	100	90	80	70	60	50	40	30	20	10	0
B.	0	10	20	30	40	50	60	70	80	90	100

8.2.1.3 Three plant combination study

To determine the combined interaction between the three extracts, a stock of 80 mg/ml extract in acetone was prepared. This concentration was increased to take into account the extra dilution factor of the third extract. For each of the eleven ratios, a total of 100 µl was prepared by combining various volumes of each extract (Table 8.3). In each combination, the volume of extract A and B were altered with extract C being kept constant (20 µl). These ratios were based on the two plant combination studies (Section 8.2.1.2). Once the combination was prepared, 100 µl was plated out in duplicate into a 96-well microtitre plate and seven 1:1 serial dilutions were prepared in water such as to determine the MIC for each ratio (Section 4.2.2.4). This constituted one experiment from which an isobologram could be generated to determine the type of interaction that existed between the three extracts. To determine the effect of varying concentrations of extract C, three concentrations were chosen to observe how the MIC values and isobolograms were altered. These concentrations were determined from the MIC values of the individual extracts (Table 4.1), namely MIC; half the MIC and double the MIC values, such that the values and concentrations in Table 8.4 and Table G1 were used. By altering the concentration of extract C, three isobolograms were generated for each triple plant

combination and each isobologram overlaid for easier comparison. This protocol was repeated for each possible combination such that 9 isobolograms were generated to determine the influence of varying concentrations of each extract in the triple combination regimes used by the traditional healers.

Table 8.3: Volumes of plant extracts to prepare the varying ratios used to determine the MIC for the triple combination.

Extract combination number	Extract volumes (µl)		
	Extract A	Extract B	Extract C
1	0	100	0
2	8	72	20
3	16	64	20
4	24	56	20
5	32	48	20
6	40	40	20
7	48	32	20
8	56	24	20
9	64	16	20
10	72	8	20
11	100	0	0

Table 8.4: MIC values of the three extracts used in the triple combination experiments (mg/ml).

Plant extract	Concentrations (µg/ml) used		
	MIC	½ MIC	2 x MIC
B. salicina	11	5.5	22
S. cordatum	2	1	4
O. sphaerocarpa	1	0.5	2

8.2.1.4 Data analysis

The two combination study isobolograms were constructed to determine the interaction between the plant extracts as previously described (Section 5.2.2; Figure 5.4). The isobolograms were constructed by plotting the following equation:

$$(X : Y) = \frac{\text{MIC of extract B in combination}}{\text{MIC of extract B alone}} : \frac{\text{MIC of extract A in combination}}{\text{MIC of extract A alone}}$$

The fractional inhibitory concentration (FIC) was also calculated to determine the strength of the interaction using the equation (Bell, 2005):

$$\text{FIC} = \frac{\text{MIC of extract A in combination with extract B}}{\text{MIC of extract A alone}}$$

The sum of the FIC values (ΣFIC) was calculated by adding all the FIC values for all the combination ratios and averaged between three individual experiments (Appendix F). The proposed definition by Berenbaum (1978) was used to determine the type and strength of the interaction; where a sum of 1 is an additive interaction, <1 a synergistic interaction, and >1 an antagonistic interaction.

The triple plant combination data was analysed in a similar manner to the two plant combination experiments by using the two plants added in different ratios and keeping the third plant as a constant. Three isobolograms were constructed from the data of the three experiments where the initial concentrations were altered (MIC, half or twice the MIC). These were overlaid and plotted together with the isobologram generated from the two plant combination experiments (i.e. without the third extracts).

8.2.2 Toxicity combination studies

8.2.2.1 Two plant combination study

Transformed human kidney epithelial cells were maintained and tryspinized as described in Section 6.2.2.3. A cell suspension of 0.5 million cells/ml (4.5 x10^5 cells/well) was used to determine cellular viability. The different combinations, prepared in DMSO, as illustrated in Table 8.5 and their serial 1:1 dilutions were added to human kidney epithelial cells, incubated and analysed as described in Section 6.2.2.4. Each combination was plated in triplicate in a 96-well microtitre plate and each combination repeated three times. The concentrations

selected for used in these ccombination experiments were bases on the IC_{50} values of the individual extracts (Table 6.1).

Table 8.5: Concentrations of extract used in combination experiments for determining cellular viability, (a) *O. sphaerocarpa* and *B. salicina*, (b) *O. sphaerocarpa* and *S. cordatum*, (c) *B. salicina* and *S. cordatum*.

Combination	Plant extract	Concentrations of extract (µg/ml)							
a	*O. sphaerocarpa*	0	1	5	10	20	40	50	80
	B. salicina	400	200	100	50	25	10	1	0
b	*O. sphaerocarpa*	0	1	5	10	20	40	50	80
	S. cordatum	100	80	50	20	10	5	1	0
c	*B. salicina*	0	1	10	25	50	100	200	400
	S. cordatum	100	80	50	20	10	5	1	0

8.2.2.2 Data analysis

The IC_{50} values of the different combinations were determined by plotting log sigmoid dose-response curves using the Enzfitter® program. Thereafter isobolograms were constructed and interpreted (Section 8.2.1.5).

8.2.2.3 Three plant combination study

To determine the type of interaction between the three extracts in combination (1:1:1), an extract concentration of 66.67 µg/ml of each of the extracts was prepared in DMSO, before being mixed in a 1:1:1 ratio. The highest concentration of the combination was 200.01 µg/ml. To test cellular viability, the extract combination was cultured with human kidney epithelial cells as described in Section 6.2.2.4. The experiment was repeated three times and the IC_{50} values determined for each experiment using the Enzfitter® program (version 1.05) upon plotting log sigmoid dose-response curves.

8.3 Results

8.3.1 Antimicrobial combination studies

The antibacterial activity of *S. cordatum*, *O. sphaerocarpa* and *B. salicina* combined in a 1:1:1 ratio (MIC value of 0.44 mg/ml) on *E. coli*, was similar to the effect of *S. cordatum* and *O. sphaerocarpa* (MIC = 0.33 mg/ml) combined in a 1:1 ratio; in comparison with the

combination between *B. salicina* and *O. sphaerocarpa* (MIC = 1.67 mg/ml) and *B. salicina* and *S. cordatum* (MIC = 1.00 mg/ml) being less effective on *E. coli* (Figure 8.2). The interaction between *S. cordatum* combined with *O. sphaerocarpa* resulted in an overall synergistic interaction (ΣFIC value of 0.56 ± 0.16) when all the individual FIC values were averaged. *Syzygium cordatum* and *B. salicina* also displayed a synergistic interaction with ΣFIC value of 0.73 ± 0.04, as well as *B. salicina* and *O. sphaerocarpa* (ΣFIC value of 0.89 ± 0.13) (Figure 8.3). The combination between *B. salicina* and *O. sphaerocarpa*, though giving a sum FIC depicting synergy, was more additive/ antagonistic when viewing the isobologram itself as most of the points lie above the line marking additivity. At the 1:1 concentration ratio and those ratios where there was a higher concentration of *B. salicina* compared to *O. sphaerocarpa*, the interaction was more antagonistic (Figure 8.4, Appendix F).

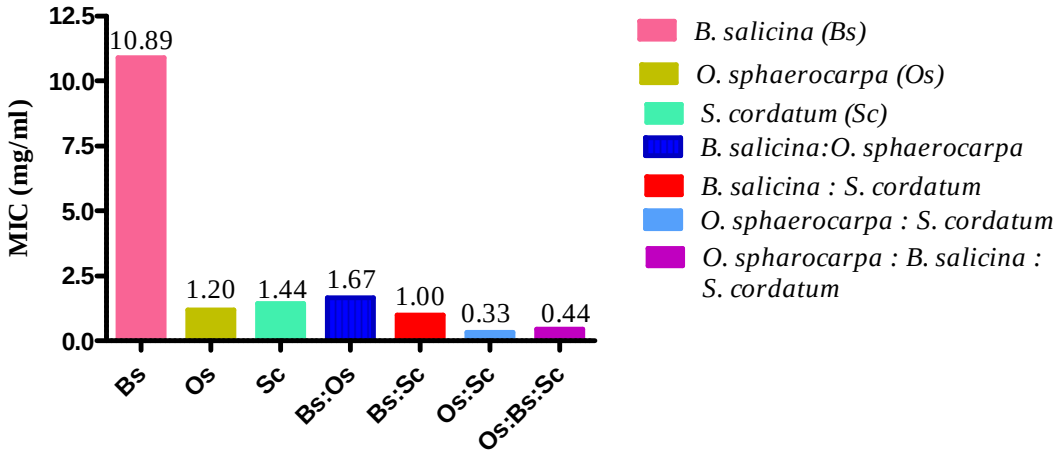


Figure 8.2: The effect on MIC for the different extract combinations against *E. coli*.

The inclusion of a third extract to the combinations to generate the triple combination isobolograms altered the resultant interactions between the different extracts, depending on the concentration of the third extract that being added. *S. cordatum* and *O. sphaerocarpa*, which had given a synergistic interaction (ΣFIC value of 0.56 ± 0.16), retained the synergy with the addition of *B. salicina* at the three concentrations, MIC, ½ MIC and 2x MIC with the ΣFIC values of 0.63, 0.49 and 0.24, respectively (Figure 8.4). The combination between *B. salicina* and *O. sphaerocarpa* resulted in an additive/antagonistic interaction (although the ΣFIC depicted synergy with a value of 0.89 ± 0.13). However, upon the addition of *S. cordatum*, the

interaction clearly became additive/antagonistic with ΣFIC values of 1.81, 0.83 and 1.11 for the MIC, ½ MIC and 2x MIC concentrations, respectively. *B. salicina* and *S. cordatum* continued exhibiting synergy/additivity even upon the addition of *O. sphaerocarpa* with ΣFIC values of 0.94, 0.67 and 0.54 for the MIC, ½ MIC and 2x MIC concentrations (Figure 8.5; Appendix G).

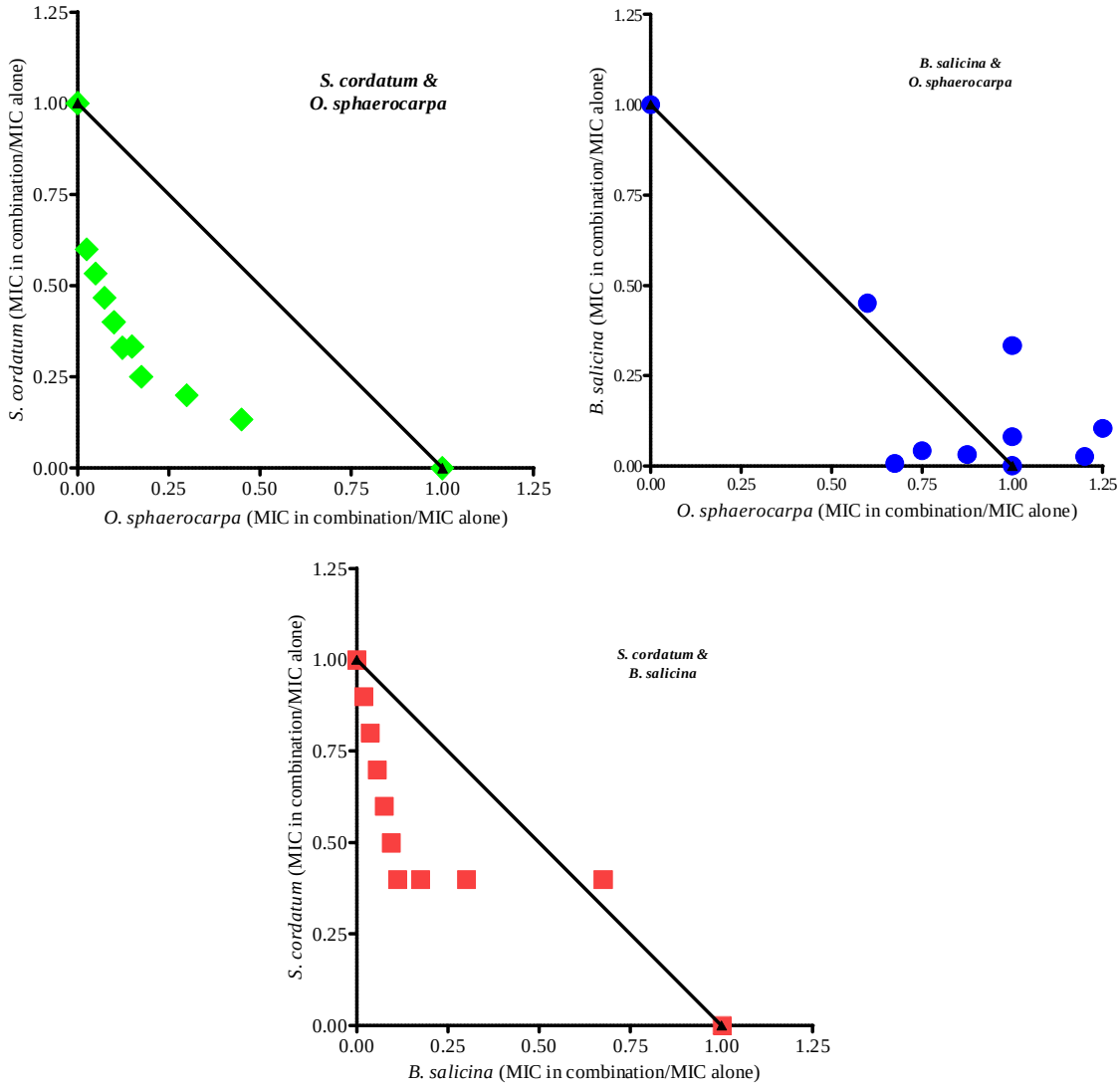


Figure 8.3: Interaction between two plants used in combination for the treatment of *E. coli*-associated diarrhoea.

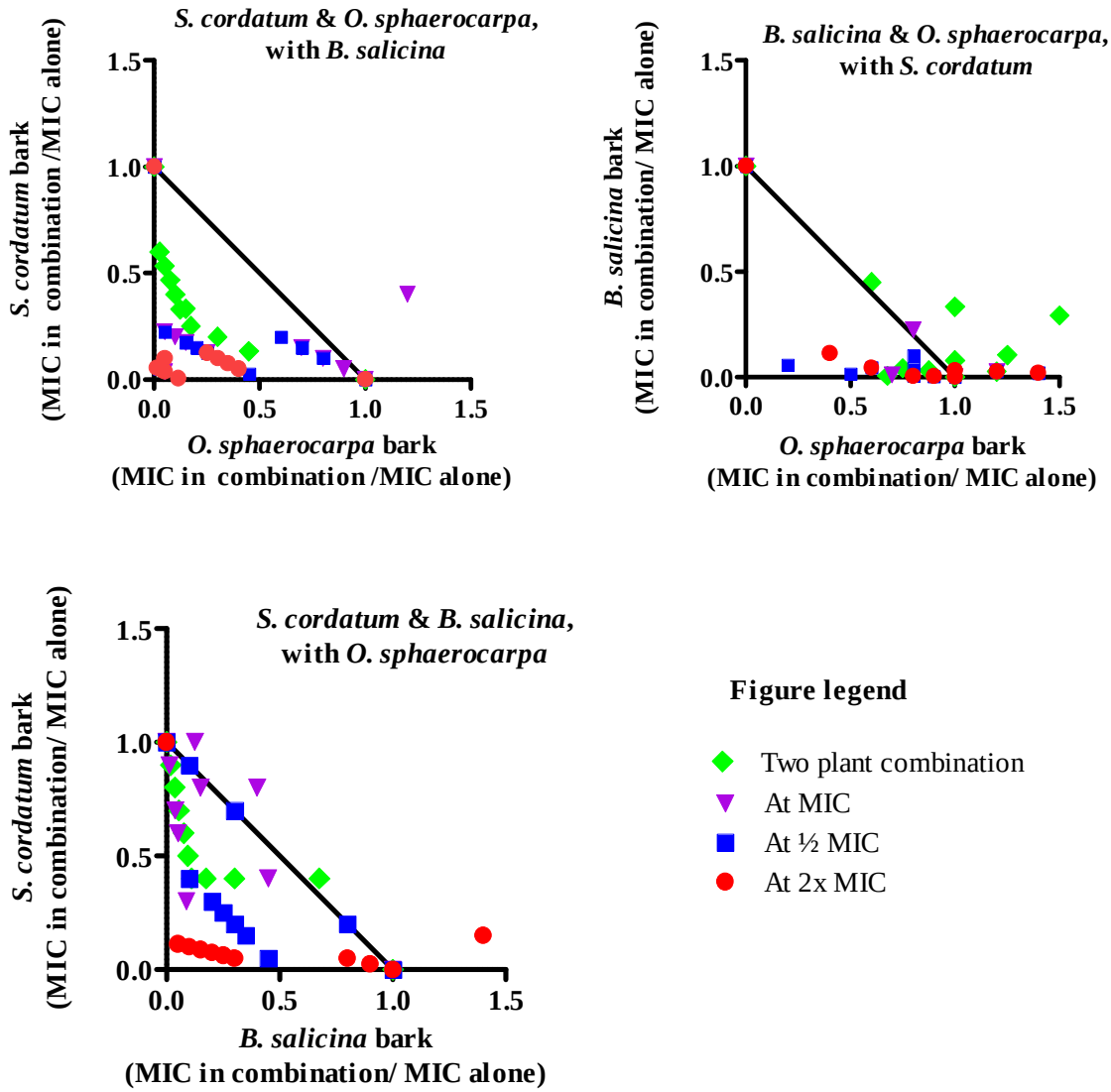


Figure 8.4: The interaction between the three plants in combination, with the third plant at constant concentration, either MIC, ½ MIC or 2x MIC. The isobologram for the two plant combination has been overlayed for comparison.

8.3.2 Toxicity results

The interaction between the least toxic of the extracts, *B. salicina* and *S. cordatum*, tested against transformed human kidney epithelial cells was synergistic (ΣFIC value of 0.46 ± 0.01) (Figure 8.5). This means that the overall toxicity of the extracts was increased when the two were combined in all the ratios tested. *Ozoroa sphaerocarpa* and *S. cordatum* displayed mostly additive/antagonistic interactions (ΣFIC value of 1.48 ± 0.25), depending on the ratio

of the combination (Figure 8.6; Appendix H). A similar interaction was observed between *O. sphaerocarpa* and *B. salicina* (ΣFIC = 1.02 ± 0.27), with some combinations resulting in synergistic/antagonistic interactions (Figure 8.6; Appendix H). The three plants combined together in a 1:1:1 ratio was non-toxic against kidney epithelial cells with an IC_{50} value of 155.76 ± 11.86 µg/ml. This meant that the safety of the combination was increased as the *O. sphaerocarpa* and *S. cordatum* were individually toxic human kidney epithelial cells and *B. salicina*, non-toxic (Table 6.1).

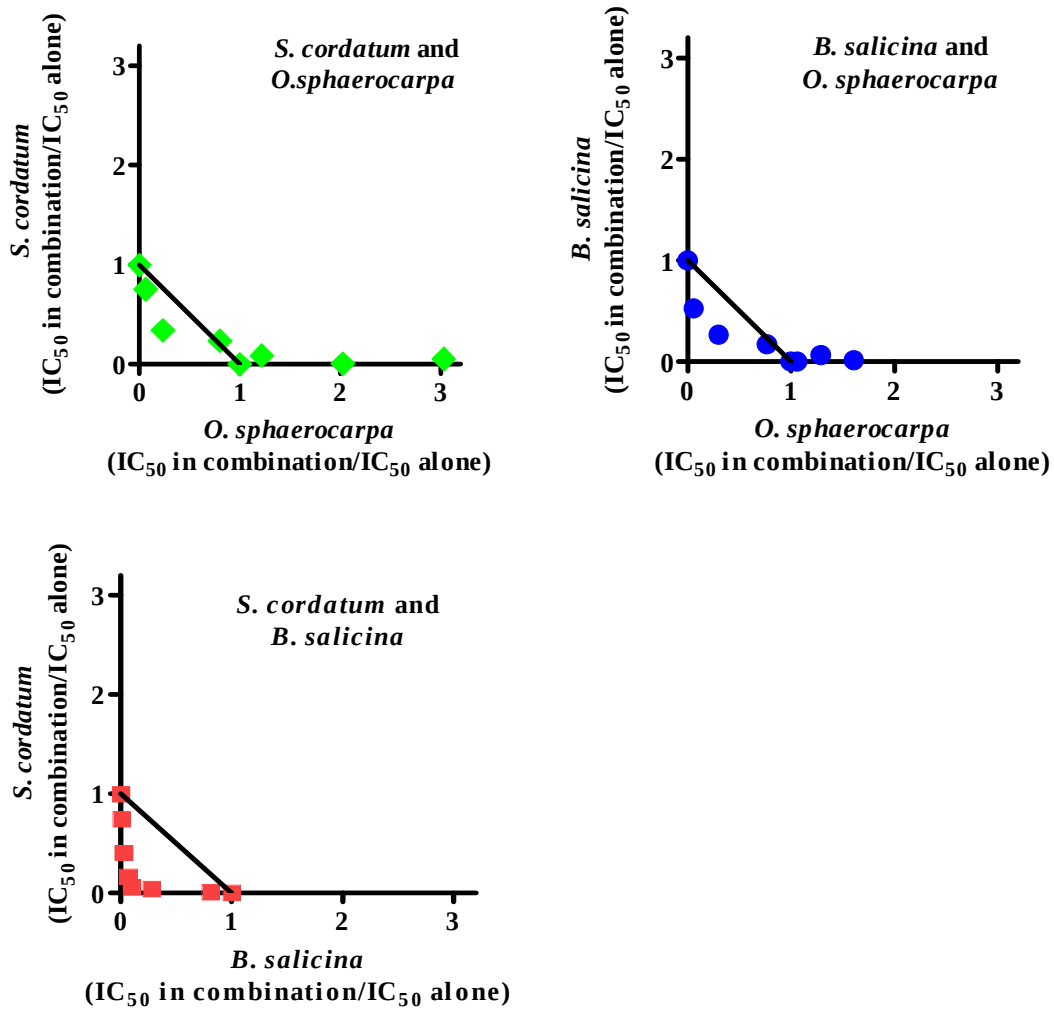


Figure 8.5: The interactions between the different extracts tested against human kidney epithelial cells.

8.4 Discussion

The combined effect of the plants against *E. coli* was predominantly synergistic, regardless of the ratio and plant combination. The combination between *B. salicina* and *O. sphaerocarpa* gave an overall synergistic interaction (ΣFIC value of 0.89 ± 0.13) whereas when viewing the isobologram, most of the points lie in the additive/antagonism area (Figure 8.3). The 1:1 combination of these plants had a lower MIC value (1.67 mg/ml) compared to *B. salicina* alone, whose MIC value was 10.89 mg/ml (Figure 8.2). This is also evidence of synergy in this combination, but it was the least effective in inhibiting the growth of *E. coli*. The addition of different concentrations of *S. cordatum* to this combination resulted in a more antagonistic interaction (Figure 8.4), especially at the MIC concentration of *S. cordatum* (Appendix G). With regards to toxicity, this combination gave an overall additive interaction with some ratios interacting synergistically and antagonistically (Figure 8.6; Appendix H) when tested against transformed human kidney epithelial cells. When dealing with toxicity studies for combination treatments, the desired effect is antagonism because this interaction results in reduced toxicity of either of the extracts. The ratios that resulted in antagonism were the higher concentrations of *O. sphaerocarpa*, compared to *B. salicina*, such as those concentrations between 20 and 50 μg/ml (Table H3). Between 1 and 10 μg/ml of *O. sphaerocarpa*, the predominant interaction was synergism.

When combining drugs in therapy, synergism is the most desired effect because this mechanism of interaction results in more effectiveness of the combination than the individual drugs (Berenbaum, 1978). *B. salicina* and *S. cordatum* displayed a strong synergistic interaction in inhibiting the growth of *E. coli* (Figure 8.3). This is also evident in the 1:1 combination of these plants (Figure 8.2), where the MIC value of both extracts was significantly reduced when added together. The antimicrobial activity of *B. salicina* and *S. cordatum* was improved upon the addition of *O. sphaerocarpa* (Figure 8.4). The relationship between *B. salicina* and *S. cordatum* with respect to toxicity was strongly synergistic (Figure 8.5), indicating increased toxicity of the combination.

The combination between *S. cordatum* and *O. sphaerocarpa* was synergistic with regards to antimicrobial activity (Figure 8.3). The 1:1 combination of these plants greatly lowered their individual MIC values (Figure 8.2). This did not change even with the addition of *B. salicina*

which resulted in even stronger synergism (Figure 8.2; Figure 8.4). The toxicity results of this combination were antagonistic (Figure 8.5). Therefore, the more favourable two plant combination is that between *O. sphaerocarpa* and *S. cordatum* (ΣFIC value of 1.48 ± 0.25) for safer toxicity, which also gave the best antimicrobial activity (ΣFIC = 0.56 ± 0.16).

It is noteworthy that combinations involving *B. salicina* were not favourable, both with reference to toxicity and antimicrobial activity, with the exception of the antimicrobial activity of *B. salicina* in combination with *S. cordatum* which displayed the desired synergistic interaction (Figure 8.3). The inclusion of *B. salicina* in the combination might serve another purpose other than its inhibitory effect on diarrhoeal pathogens. This is shown by its poor antimicrobial activity against the five microbial strains tested in this study (Table 4.1). However, on its own, it displayed the best toxicity profile of the three plants, with the highest IC_{50} value against human kidney epithelial cells (Table 6.1) as well as being non-toxic to red blood cells (Table 5.2). It is interesting to note that *B. salicina* is traditionally used to treat diarrhoea as well as wounds or injuries (Neuwinger, 1996; Venter and Venter, 2002), however, in this study; it did not inhibit the growth of any of the pathogens tested. It is possible that it mediates its activity in these areas through another mechanism of action such as acting as an adsorbent, like isphaghula husk for an anti-diarrhoeal effect, or fibroblast growth stimulation for wound healing.

Syzygium species have, for a long time, been reported to possess anti-diarrhoeal properties and are used in many countries for the management of diarrhoea. As an example, *S. samarangense* is used by the Philippines in the treatment of diarrhoea (Ghayur *et al*., 2006) and in Southern Africa, *S. cordatum* is used for the treatment of stomach troubles, cold and fever and diarrhoea (Amusan *et al*., 2002; Samie *et al*., 2005; Mathabe *et al*., 2006). In the present investigation, the anti-*E. coli* activity of *S. cordatum* in combination with either *B. salicina* or *O. sphaerocarpa* is reported. Alone, *S. cordatum* was able to inhibit microbial growth of *E. coli* (Table 4.1), and in combination, acted synergistically to inhibit *E. coli* (Figure 8.3). The activity of this plant is supported by reports on the antimicrobial / anti-*E. coli* / anti-diarrhoeal effects of *Syzygium* species (Hammer *et al*., 1999; Dorman and Deans, 2000; Djoukeng *et al*., 2005; Samie *et al*., 2005; Mathabe *et al*., 2006). The anti-diarrhoeal activity of *S. samarangense* has been attributed to the presence of flavonoids which have been

found to possess relaxant activity on isolated rabbit jejunum, mediated through the blockade of calcium influx (Ghayur *et al*., 2006), as well as possess antibacterial properties (Palombo, 2006). This study has also found an appreciable content of phenolics and flavonoids isn both leaves and bark of this species which may be responsible for the anti-*E. coli* activity.

Ozoroa sphaerocarpa displayed good antimicrobial activity against all the micro-organisms tested in this study (Table 4.1). It also demonstrated good activity against *E. coli* when used alone, with an MIC value of 1.20 mg/ml (Figure 8.2). Mathabe *et al*. (2006) demonstrated anti-*E. coli* activity of *O. insignis* with an MIC of 0.078 mg/ml. Apart from being used to treat diarrhoea, *O. insignis* is used to treat tapeworm and hookworm (Mølgaard *et al*., 2001; Rea *et al*., 2003). When in combination, *O. sphaerocarpa* was able to suppress *E. coli* growth effectively, when combined with *S. cordatum*.

The triple combination (1:1:1), as traditionally used was found to have acceptable toxicity levels, that is 155.76 ± 11.86 μg/ml. The antimicrobial activity of the combination between *O. sphaerocarpa* and *S. cordatum* was also more favourable, displaying synergism, which was also improved by the addition of increasing concentrations of *B. salicina*, resulting in a more synergistic interaction (Figure 8.4). Though the traditional remedy involves using all three plants in equal ratios, this investigation has shown that changing the concentrations of the extracts may result in improved activity, as illustrated by the increase in synergy upon addition of a higher concentration of the third extract (Figure 8.4).

Work carried by other researchers has shown the presence of synergism between different plant combinations (Boik, 2001; Kamatou *et al*., 2006; Okusa *et al*., 2007). In addition, synergistic/ additive effects were exhibited by extracts of *Kola nitida* seed in combination with some fluoroquinolines when tested against *E. coli*. This is beneficial in delaying possible resistance to fluoroquinolines and is essential information to avoid therapeutic failure when treating a patient who has initially received *K. nitida* before fluoroquinolines are administered (Ibezim *et al*., 2006). Another study, using *Cordia gilletii* De Wild with tetracycline and streptomycin, gave additive and synergistic interactions when tested against *E. coli* and *Staphylococcus aureus* (Okusa *et al*., 2007). The presence of synergy between plant extracts or between plants and standard microbial drugs emphasizes the need for further research into

combination work due to the increase risk of patients developing resistance to the agents when used as monotherapy. In the present study, all possible interactions have been displayed at various concentration ratios; however, interaction with standard antimicrobial agents has not been investigated. There is need to investigate the interaction of these plants with standard antimicrobials and this was outside the scope of this study as our aims were to validate the effectiveness of the three plants in combination against a diarrhoea causing pathogen.

Plants have been effectively used to combat diarrhoea for centuries, especially in the African setting. Studies on anti-diarrhoeal efficacy of some of these plants have shown variable mechanisms of action. Some of the anti-diarrhoeal activity is mediated through the action on diarrhoea pathogens like *E. coli*, *Salmonella* spp and *Shigella* spp (Alanís *et al*., 2005), while others play an important role as smooth muscle relaxants as well as by inhibiting prostaglandin synthesis (Agunu *et al*., 2005; Gutiérrez e*t al*., 2007). Others do not appear to interfere with any of the above, but are still excellent anti-diarrhoeal agents which stimulate water re-absorption or reduction of intraluminal fluid accumulation (Gutiérrez e*t al*., 2007).

Though not all possible modes of action have been investigated in the present study, it can be concluded that *S. cordatum* and *O. sphaerocarpa* do possess anti-diarrhoeal activity mediated by inhibiting the growth of *E. coli*. The combination of the two is also active at all concentrations tested. *B. salicina*, on the other hand, though contributing some activity, lacked anti-*E. coli* activity, but its anti-diarrhoeal activity may be due to some other mechanisms, as previously discussed.

The results of the combination experiments support the traditional use of these three plants in combination for the treatment of diarrhoea and also prove that traditional medicine is a reliable source of knowledge for the development of new drugs.

CHAPTER 9: GENERAL CONCLUSIONS AND RECOMMENDATIONS

9.1 CONCLUSIONS

- This study highlights the great potential of Swazi medicinal plants in the treatment of malaria as the leaves of *T. phanerophlebia*, bark of *S. cordatum*, bark of *B. salicina* and *P. meyeri* (whole plant) exhibited antimalarial activity below 10μg/ml. However, when tested in combination with quinine, these extracts showed predominantly antagonistic interactions at the different ratios. This could result in treatment failure if the plants are used in combination with quinine in the treatment of malaria. Therefore great caution should be taken when using these plants for the traditional treatment of malaria, whilst on standard antimalarial drugs or visa versa.

- Over fifty percent of the extracts tested did not show cytotoxicity to human kidney epithelial cells and none showed significant red blood cell lysis, with *H. acutatum* (roots), *G. densa* (whole plant), *B. salicina* (bark), *T. phanerophlebia* (bark), *B. setifera* (leaves), *Z. mucronata* (fruit) and *B. setifera* (flower) being the least cytotoxic. Of the cytotoxic plants, it should be taken into consideration that the *in vitro* results are not necessarily indicative of what could happen *in vivo*.

- The fruit of *O. sphaerocarpa* was the most active against all the microbial pathogens tested with the lowest MIC values of 1.00 μg/ml against *S. epidermidis*, 20μg/ml against *S. aureus* and 70 μg/ml against *K. pneumoniae*. The bark of *F. glumosa* and the fruit of *D. cinerea* were the most active against *E. coli* with a MIC value of 0.25 mg/ml. The leaves of *T. emetica* and *Z. mucronata*, the flower of *L. intermedia* and leaves of *R. communis* were the most active against *C. albicans* with MIC values of 0.22, 0.23, 0.25 and 0.29 mg/ml, respectively.

- The combined effect of *S. cordatum*, *O. sphaerocarpa* and *B. salicina* against *E. coli* was predominantly synergistic, even though there was some additive/ antagonistic interaction between some of the combination ratios. The best two plant combination was that between *S. cordatum* and *O. sphaerocarpa* which was also relatively non-toxic against human kidney epithelial cells. The results of the combination experiments provide scientific support for the traditional use of these three plants in combination for the treatment of diarrhoea.

- The extracts studied were found to be good sources of anti-oxidants as they displayed good activity to scavenge free radicals, chelate ferrous ions and inhibit lipid peroxidation inhibition. Some of the extracts, such as the leaves and bark of *T. phanerophlebia*, leaves of *S. cordatum*, fruit of *Z. mucronata* and the flower, stem/root and leaves of *B. setifera* were also found to possess a relatively high content of phenolics and flavonoids which may be responsible for the anti-oxidant activity. Their medicinal properties may also be linked to their anti-oxidant activity.

- The majority of the UPLC and HPTLC profiles of the plants were very complex, indicating the presence of many compounds in the plants which could be responsible for their pharmacological activities. There were also many similarities between different parts of the same plant

9.2 RECOMMENDATIONS

- The fruit of *O. sphaerocarpa* exhibited antimicrobial potential, especially against *Staphylococcal* pathogens; therefore from this study it can be recommended that further antimicrobial studies be conducted using the fruit extract. In addition, the fruit pericarp and seeds must be separated to determine the active part, as well as to determine if the active part is indeed responsible for the cytotoxicity observed for the whole fruit. The bio-autography results showed a wide band that inhibited the growth of *E. coli* when the fruit components were separated by TLC. This should be isolated, identified and further tested for possible development.

- Antimalarial activity of the extracts should be further determined using an assay that targets the sexual stages of the parasite (gametocytes) as they may have activity against this stage of the parasite life cycle. If active at this stage, they could interfere with transmission of the parasite from one host to the next. Further assays should be conducted to evaluate the mode of action of the active extracts by employing several techniques like inhibition of haemozoin formation.

- Some plant extracts displayed cytotoxicity, however, it should be taken into consideration that the *in vitro* results are not necessarily indicative of what could happen *in vivo*, therefore the panel of human cells on which the extracts are tested should be expanded to validated if they are indeed toxic, further, the most active

and least toxic extract should be taken in to an in vivo model to determine the toxicity of its breakdown products/metabolites.

- The most effective plants in all the assays employed should be further tested for interactions with standard drugs so as to determine the effect that would arise from using them concurrently with drugs used in clinical practice. The present study has already established antagonistic interactions of some of the extracts when combined with quinine, but it is not known what would happen were the extracts combined with other antimalarial drugs such as artemisinin or lumefantrine or antimicrobial drugs such as ciprofloxacin, amphotericin B, doxycycline or clindamycin.

REFERENCES

Abreu PJM, Liu Y. Ozoroalide, a new macrolide from *Ozoroa insignis*. Fitoterapia 2007; 78: 388-389.

Abu-Mustafa EA, El-Tawil BAH, Fayez MBE. Constituents of local plants-IV. *Ficus carica* L., *F. sycomorus* L. and *F. salicifolia* L. leaves. Phytochemistry 1964; 3: 701 to 703.

Adeniji KO, Amusan OOG, Dlamini PS, Enow-Orock EG, Gamedze ST, Gbile ZO, Langa AD, Makubu LP, Mahunnah RLA, Mshana RN, Sofowa A, Vilane MJ. 2000. Traditional medicine and pharmacopoeia contribution to ethnobotanical and floristic studies in Swaziland. Scientific, Technical and Research Commission of the Organization of African Unity (OAU/STRC).

Adeyibi A, Adaikan PG, Prasad RNV. Tocolytic and toxic activity of papaya seed extract on isolated rat uterus. Life Sciences 2003; 74: 581-592.

Afolayan AJ, Meyer JJM. The antimicrobial activity of 3,5,7-trihydroxyflavone isolated from the shoots of *Helichrysum aureonitens*. Journal of Ethnopharmacology 1997; 57: 177-181.

Agunu A, Yusuf, S, Andrew GO, Zezi AU, Abduranhman EM. Evaluation of five medicinal plants used in diarrhoea treatment in Nigeria. Journal of Ethnopharmacology 2005; 101: 27-30.

Ah Kee NL, Mnonopi N, Davids H, Naudé RJ, Frost CL. Antithrombotic/anticoagulant and anticancer activities of selected medicinal plants from South Africa. African Journal of Biotechnology 2008; 7: 217-223.

Al Fatimi M, Wurster M, Schröder G, Lindequist U. Antioxidant, antimicrobial and cytotoxic activities of selected medicinal plants from Yemen. Journal of Ethnopharmacology 2007; 111: 657-666.

Alam MS, Kaur G, Ali A, Hamid H, Ali M, Athar M. Two new bioactive oleanane triterpene glycoside from *Terminalia arjuna*. Natural Product Research 2008; 22: 1285-1294.

Alanís AD. Calzada F, Cervantes JA, Torres J, Ceballos GM. Antibacterial properties of some plants used in Mexican traditional medicine for the treatment of gastrointestinal disorders. Journal of Ethnopharmacology 2005; 100: 153-157.

Allen HJ, Johnson EAZ. The isolation of lectins on acid-treated agarose. Carbohydrate Research 1976; 50: 121-131.

Al-Rehaily AJ, Albishi OA, El-Olemy MM, Mossa JS. Flavonoids and terpenoids from *Helichrysum forskahlii*. Phytochemistry 2008; 69: 1910-1914.

Alternative Gardening. www.gardening.co.za/alternative-gardening-medicinal.html, accessed on the 11th April, 2006.

Aluka.2006.
(http://www.aluka.org/action/showMetadata?doi=10.5555/AL.AP.FLORA.FZ6637&pgs=), accessed 13th February 2008.

Ames BN, Shigenaga MK, Hagen TM. Oxidant, antioxidants and the degenerative diseases of aging. Proceedings of the National Academy of Sciences of the United States of America 1993; 90: 7915-7922.

Amusan OOG (Ed). April 2007. Proceedings of the workshop on the integration of traditional medicine with convention medicine in the health delivery systems in Swaziland. Kwalusini Swaziland.

Amusan OOG, Dlamini PS, Msonthi JD, Makhubu LP. Some herbal remedies from Manzini region of Swaziland. Journal of Ethnopharmacology 2002; 79: 109-112.

Amusan OOG, Sukati NA, Hlophe FG. Herbal Remedies from Shiselweni Region of Swaziland. Phytotherapeutics, Recent Progress in Medicinal Plants 2005; 10: 451-471.

Amusan OOG, Sukati NA, Dlamini PS, Sibandze FG. Some Swazi phytomedicines and their constituents. African Journal of Biotechnology 2007; 6: 267-272.

Anderson DMW, Bell PC. The composition and properties of the gum exudates from *Terminalia sericea* and *T. superba*. Phytochemistry 1974; 13: 1871-1874.

Annan K. Houghton PJ. Antibacterial, antioxidant and fibroblast growth stimulation of aqueous extracts of *Ficus asperifolia* Miq. and *Gossypium arboreum* L., wound-healing plants of Ghana. Journal of Ethnopharmacology 2008; 119: 141-144.

Ao C, Li A, Elzaawely AA, Xuan TD, Tawat S. Evaluation of antioxidant and antibacterial activities of *Ficus microcarpa* L. fil. extract. Food Control 2008; 19: 940 -948.

Asase A, Oteng-Yeboah AA, Odamtten GT, Simmonds MSJ. Ethnobotanical study of some Ghanaian anti-malarial plants. Journal of Ethnopharmacology 2005; 99: 273-279.

Astrographics. com. 2002.
http://www.astrographics.com/GalleryPrintsIndex/GP2118.html, accessed on the 25th April 2009.

Azebaze AG, Dongmo AB, Meyer M, Ouahouo BM, Valentin A, Laure Nguemfo E, Nkengfack AE, Vierling W. Antimalarial and vasorelaxant constituents of the leaves of *Allanblackia monticola* (Guttiferae). Annals of Tropical Medicine and Parasitology 2007; 101: 23-30.

Bah S, Jäger AK, Adsersen A, Diallo D, Paulsen BS. Antiplasmodial and $GABA_A$-benzodiazepine receptor binding activities of five plants used in traditional medicine in Mali, West Africa. Journal of Ethnopharmacology 2007; 110: 451-457.

Bahorun T, Soobratte MA, Luximon-Ramma V, Aruoma OI. Free radicals and Antioxidants in Cardiovascular Health and Disease. Internet Journal of Medical Update 2006; 2: http://www.geocities.com/agnihotrimed/papero5_jul-dec2006.htm.

Balint GA. Ricin: The toxic protein of castor oil seeds. Toxicology 1974; 2: 77-102.

Bandaranayake WM. 2006.Quality Control, Screening, Toxicity, and Regulation of Herbal Drugs, In: Ahmed I, Aqil F, Owais M (Eds). Modern Phytomedicine. Turning Medicinal Plants into Drugs. Wiley-VCH Verlag GmbH & Co. KGaA, Weiheim.

Bannister BA, Begg NT, Gillespie SH. 2000. Infectious Disease. 2nd edition. Blackwell Science Ltd. Osney Mead, Oxford.

Banso A, Adeyemo SO. Evaluation of antibacterial properties of tannins isolated from *Dichrostachys cinerea*. African Journal of Biotechnology 2007; 6: 1785-1787.

Becker K, Tilley L, Vennerstrom JL, Roberts D, Rogerson S, Ginsburg H. Oxidative stress in malaria parasite-infected erythrocytes: host-parasite interactions. International Journal of Parasitology 2004; 34: 163-189.

Bell A. Antimalarial drug synergism and antagonism: Mechanistic and clinical significance. FEMS Microbiology Letters 2005; 253: 171-184.

Benherlal PS, Arumughan C. Chemical composition and *in vitro* antioxidant studies on *Syzygium cumini*. Journal of the Science of Food and Agriculture 2007; 87: 2560-2569.

Berenbaum MC. What is synergy? American Society for Pharmacology and Experimental Therapeutics 1989; 41: 93-141.

Berenbaum, MC. A method for testing for synergy with any number of agents. The Journal of Infectious Diseases 1978; 137: 122–130.

Bessong PO, Obi CL, Andréola M, Rojas LB, Pouységu L, Igumbor E, Meyer JJM, Quideau S, Litvak S. Evaluation of selected South African medicinal plants for inhibitory properties against human immunodeficiency virus type 1 reverse transcriptase and integrase. Journal of Ethnopharmacology 2005; 99: 83-91.

Bhattaram VA, Graefe U, Kohlert C, Veit M, Derendorf H. Pharmacokinetics and bioavailabity of herbal medicinal products. Phytomedicine 2002; 9, Supplement III: 1-33.

Bohlmann F, Mahanta PK, Zdero C. Neue chalkon-derivative aus Sudafrikanisschen *Helichrysryum*-arten. Phytochemistry 1978; 17: 1935-1937.

Boik J. Natural Compounds in Cancer Therapy. 2001. Oregon Medical Press. LCC, USA.

Bouayed J, Piri K, Rammal H, Dicko A, Desor F, Younos C, Soulimani R. Comparative evaluation of the antioxidant potential of some Iranian medicinal plants. Food Chemistry 2007; 104: 364-368.

Boucher AN, Tam VH. Mathematical formulation of additivity for antimicrobial agents. Diagnostic Microbiology and Infectious Disease 2006; 55: 319-325.

Boutayeb A. The double burden of communicable and non-communicable diseases in developing countries. Transactions of the Royal Society of Tropical Medicine and Hygiene 2006; 100: 191-199.

Bringmann G, Saeb W, Assi LA, François G, Sankara Narayanan AS, Peters K, Peters EM. Betulinic acid: isolation for *Triphyophyllum peltatum* and *Ancistrocladus heyneanus*,

antimalarial activity, and crystal structure of the benzyl ester. Planta Medica 1997; 63: 255-257.

Brooks GF, Carroll KC. 2007. Bacteriology. In: Brooks GF, Carroll KC, Butel JS, Morse SA. (Eds) Jawetz, Melnick, & Adelberg's Medical Microbiology, 24th edition. McGraw-Hill Companies Inc.

Buenz EJ, Tillner JE, Limburg P, Bauer A. Antibacterial properties and toxicity of *Atuna racemosa* extract depend on kernel maturity. Journal of Ethnopharmacology 2007; 111: 592-597.

Bunnett NW, Lingappa VR. 2006. Gastrointestinal Disease. In: McPhee SJ, Ganong WF. Pathophysiology of Disease, 5th edition. McGraw-Hill Companies Inc.

Büyükokuroğlu ME, Taysi S, Polat F, Göçer F. Mechanism of the beneficial effects of dantrolene sodium on the ethanol-induced acute gastric mucosal injury in rats. Pharmacological Research 2002; 45: 421-424.

Candy HA, McGarry EJ, Pegel KH. Constituents of *Syzygium cordatum*. Phytochemistry 1968; 7: 889-890.

Canfield CJ, Pudney M, Gutteridge WE. Interactions of atovaquone with other antimalarial drugs against *Plasmodium falciparum in vitro*. Experimental Parasitology 1995; 80: 373-381.

Centres for Disease Control and Prevention. 2006. http://www.cdc.gov/malaria/biology/life_cycle.htm, accessed on the 7th August, 2009.

Chambers HF, Deck DH. Chapters 43/44 and 46. In: Katzung BG. Basic and Clinical Pharmacology, 10th edition. McGraw-Hill Companies, Inc. United States of America.

Chan TYK, Tomlinson B, Critchley JAJH. Aconitine poisoning following the ingestion of Chinese herbal medicines: a report of eight cases. Internal Medicine Journal 1993; 23: 268-271.

Chang CW, Wu TS, Hsieh YS, Kuo SC, Chao PD. Terpenoids of *Syzygium formosanum*. Journal of Natural Products 1999; 62: 327-328.

Chart H. 2007. *Escherichia* urinary tract infection; travellers' diarrhea, haemorhagic colitis; haemolytic uraemic syndrome. In: Greenwood D, Slack R, Peutherer J, Barer M. (Eds) Medical Microbiology, 7th edition. Churchill Livingstone, Elsevier.

Chen Y, Cai L, Zhao C, Xu H, Cao C, Liu Y, Jia L,Yin H, Chen C, Zhang H. Spectroscopic, stability and radical-scavenging properties of a novel pigment from *Gardenia*. Food Chemistry 2008; 109: 269 - 277.

Chen ZY, Chan PT, Ho KY, Fung KP, Wang J. Antioxidant activity of natural flavonoids is governed by number and location of their aromatic hydroxyl groups. Chemistry and Physics of Lipids 1996; 79: 157-163.

Chhabra SC, Gupta SR, Sharma ND. A new flavone from *Gardenia* gum. Phytochemistry 1977; 16: 399.

Chiang YM, Chang JY, Kuo CC, Chang CY, Kuo YH. Cytotoxic triterpenes from the aerial roots of *Ficus microcarpa*. Phytochemistry 2005; 66: 495-501.

Chiang YM, Kuo YH. Two novel α-tocopheroids from the aerial roots of *Ficus microcarpa*. Tetrahedron Letters 2003; 44: 5125-5128.

Choi HJ, Park YS, Kim MG, Kim TK, Yoon NS, Lim YJ. Isolation and characterization of the major colorantin *Gardenia* fruit. Dyes and Pigments 2001; 49: 15-20.

Chyau CC, Tsai SY, Ko PT, Mau JL. Antioxidant properties of solvent extracts from *Terminalia catappa* leaves. Food Chemistry 2002; 78: 483-488.

Clarkson C, Maharaj VJ, Crouch NR, Grace OM, Pillay P, Matsabisa MG, Bhagwandin N, Smith PJ, Folb PI. *In vitro* antiplasmodial activity of medicinal plants native to or naturalized in South Africa. Journal of Ethnopharmacology 2004; 92: 177-191.

Coker RJ, Horner P, Bleasdale-Barr K, Harris JRW, Mathias CJ. Increased gut parasympathetic activity and chronic diarrhoea in a patient with the acquired immunodeficiency syndrome. Clinical Autonomic Research 1992; 2: 295-298.

Compton RH. 1976. The Flora of Swaziland. Journal of South African Botany, Supplementary volume 11.

Constable S, Ham, A, Pirmohamed M. Herbal medicines and acute medical emergency admissions to hospital. British Journal of Clinical Pharmacology 2006; 63: 247-248.

Cosmetic Ingredient Review Expert Panel. Final report on the safety sssessment of *Ricinus Commmunis* (Castor) seed oil hydrogenated castor oil, glyceryl ricinoleate, glyceril ricinoleate SE, ricinoleic acid, potassium ricinoleate, sodium ricinoleate, zinc ricinoleate, cetyl ricinoleate, and octydodecyl ricinoleate. International Journal of Toxicology 2007; 26: 31-77.

Coulombe Jr. RA, Drew GL, Stermitz FR. Pyrrolizidine alkaloids crosslink DNA with actin. Toxicology and Applied Pharmacology 1999; 154: 198-202.

Covacci V, Torsello A, Palozza P, Sgambato A, Romano G, Boninsegna A, Cittadini A, Wolf FL. DNA oxidative damage during differentiation of HL-60 human promyelocytic leukaemia cells. Chemical Research in Toxicology 2001; 14: 1492-1497.

Cowan MM. Plant products as antimicrobial agents. Clinical Microbiology Reviews 1999; 12: 564-582.

Cremin Jr. JD, Smith DR. *In vitro* vs *in vivo* Pb effects on brain protein kinase C activity. Environmental Research 2002; 90: 191-199.

Crump A. New medicines from nature's armamentarium. Trends in Parasitology 2006; 22: 51-54.

CUBoulder Organic Chemistry. 2008. Thin layer chromatography. http://orgchem.colorado.edu/hndbksupport/TLC/TLC.html, accessed 19th February 2008.

Cunningham AB. 1993. African Medicinal Plants: Setting priorities at the interface between conservation and primary healthcare. United Nations Educational, Scientific and Cultural Organization.

da Cunha US, Vendramim JD, Rocha WC, Vieira PC. Bioactivity of *Trichilia pallida* Swartz (Meliaceae) derived molecules on Tuta absoluta (Meyrick) (Lepidoptera: Gelechiidae). Neotropical Entomology 2008; 37: 709-715.

Daddona PE, Wiesmann WP, Lambros C, Kelley WN, Webster HK. Human malaria parasite adenosine deaminase. Journal of Biological Chemistry 1984; 259: 1472-1475.

David JP, Meira M, David JM, Brandão HN, Branco A, Agra MF, Barbosa MRV, de Queiroz LP, Giulietti AM. Radical scavenging, antioxidant and cytotoxic activity of Brazilian *Caatinga* plants. Fitoterapia 2007; 78: 215-218.

Davies NW, Miller JM, Naido R Sotheeswaran S. Triterpenoids in bud exudates of Fijian *Gardenia* species. Phytochemistry 1992; 31: 159-162.

de Boer HJ, Kool A, Broberg A, Mziray WR, Hedberg I, Levenfors JJ. Antifungal and ant-bacterial activity of some herbal remedies of Tanzania. Journal of Ethnopharmacology 2005; 96: 461-469.

De Villiers A, Lestremau F, Szucs R, Gelebart S, David F, Sandra P. Evaluation of ulra performance liquid chromatography Part I. Possibilities and limitations. Journal of Chromatography A 2006; 1127: 60-69.

Deng JF. Clinical and laboratory investigations in herbal poisonings. Toxicology 2002; 181: 571-576.

Dennehy CE, Tsourounis C. 2007. Botanicals ("Herbal Medications") & nutritional supplements, In: Katzung BG (Ed), Basic and Clinical Pharmacology 10th ed. McGraw-Hill Companies, Inc. United States of America.

Desjardins RE, Canfield CJ, Haynes JD, Chulay D. Quantitative assessment of antimalarial activity *in vitr*o by a semiautomated microdilution technique. Antimicrobial Agents and Chemotherapy 1979; 16: 710-718.

Diallo D, Paulsen BS, Liljebäck THA, Michaelsen TE. Tha Malian medicinal plant *Trichilia emetica*; studies on polysaccharides with complement fixing ability. Journal of Ethnopharmacology 2003; 84: 279-287.

Diaz GM, Arruda AC, Aruda MSP, Müler A. Methoxyflavones from *Ficus maxima*. Phytochemistry 1997; 45: 1697-1699.

Dilika F, Bremner PD, Meyer JJM. Antibacterial activity of linoleic and oleic acids isolated from *Helichrysum pedunculatum*: a plant used during circumcision rites. Fitoterapia 2000; 71: 450-452.

Dinis TCP, Madeira VMC, Almeida LM. Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and peroxyl radical rcavengers. Archives of Biochemistry and Biophysics 1994; 315: 161-169.

Diplock AT. Will the 'good fairies' please proves to us that vitamin E lessens human degenerative of disease. Free Radical Research 1997; 27: 511-532.

Djeridane A, Yousfi M, Nadjem B, Boutassouna D, Stocker P, Vidal N. Antioxidant activity of some Algerian medicinal plants extracts containing phenolic compounds. Food Chemistry 2006; 97: 654-660.

Djoukeng JD, Abou-Mansour E, Tabacchi R, Tapondjou AL, Bouda H, Lonsti D. Antibacterial triterpenes from *Syzygium guineense* (Myrtaceae). Journal of Ethnopharmacology 2005; 1010: 283-286.

Dorman HJD, Deans SG. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. Journal of Applied Microbiology 2000; 88: 308-316.

Douglas MW, Bell PC. The compositon and properties of the gum exudates from *Terminalia sericea* and *T. superba*. Phytochemistry 1974; 13: 1871-1874.

Drewes SE, Mudau KE, van Vuuren SF, Viljoen AM. Antimicrobial monomeric and dimeric diterpenes from the leaves of *Helichrysum tenax* var *tenax*. Phytochemistry 2006; 76: 716-722.

Drewes SE, van Vuuren SF. Antimicrobial acylphloroglucinols and dibenzyloxy flavonoids from flowers of *Helichrysum gymnocomum*. Phytochemistry 2008; 69: 1745–1749.

Dueñas M, Pérez-Alonso JJ, Santos-Buelga C, Escribano-Bailón T. Anthocyanin composition in fig (*Ficus carica* L.) Journal of Food Composition and Analysis 2008; 21: 107–115.

Duh PD, Tu YY, Yen GC. Antioxidant activity of water extract of Harug Jyur (*Chrysanthemum morifolium* Ramat) Lebensmittel-Wissenchaft und Technologie 1999; 32: 267-277.

Duker-Eshun G, Jaroszewski JW, Asomaning WA, Oppong-Boachie F, Brøgger Christensen S. Antiplasmodial constituents of *Cajanus cajan*. Phytotherapy Research 2004;18: 128-130.

Egan TJ. Haemozoin formation. Molecular & Biochemical Parasitology 2008; 157:127-136.

Eisa MM, Almagboul AZ, Omer MEA, Elegami AA. Antibacterial activity of *Dichrostachys cinerea*. Fitoterapia 2000; 71: 324-327.

Ekong R, WarhustDC. Synergism between arteether and mefloquine or quinine in multidrug-resistant strain of *Plasmodium falciparum in vitro*. Transactions of the Royal Society of Tropical Medicine and Hygiene 1990; 84: 757-758.

El Tahir A, Satti GMH, Khalid SA. Antiplasmodial activity of selected Sudanese medicinal plants with emphasis on *Maytenus senegalensis* (Lam.) Exell. Journal of Ethnopharmacology 1999; 64: 227-233.

Eldeen IM, Van Heerden FR, Van Staden J. Isolation and biological activities of termilignan B and arjuni acid from *Terminalia sericea*. Planta Medica 2008; 74: 411-413.

Elgorashi EE, Taylor JLS, Maes A, Van Staden J, De Kimpe N, Verschaeve L. Screening of medicinal plants used in South African traditional medicine for genotoxic effects. Toxicology Letters 2003; 143: 195-207.

Eloff JN, McGaw LJ. 2006. Plant extracts used to manage bacterial, fungal and parasitic infections in southern Africa, In: Ahmed I, Aqil F, Owais M (Eds). Modern Phytomedicine. Turning Medicinal Plants into Drugs. Wiley-VCH Verlag GmbH & Co. KGaA, Weiheim.

Eloff JN. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. Planta Medica 1998; 64: 711-713.

Era-net PathoGenoMics. 2006.
www.ict-science-to-society.org/Pathogenomics/bacteria.htm, accessed on the 15th April 2009.

Evans WC. 2002. Trease and Evans Pharmacognosy 15th ed. Elsevier Limited. China.

Facino RM, Carini M, Franzoi L, Pirola O, Bosisio E. Phytochemical characterization and radical scavenger activity of flavonoids from *Helichrysum italicum* G. Don (Compositae). Pharmacological Research 1990; 22: 709-722.

Farnsworth NR, Akerele O, Bingel AS, Soejarto DD, Guo Z. Medicinal plants in therapy. Bulletin of the World Health Organisation 1985; 63: 965-981.

Farombi EO, Ogundipe OO, Uhunwangho ES, Adeyanju MA, Moody JO. Antioxidant properties of extracts from *Alchornea laxiflora* (Benth) Pax and Hoffman. Phytotherapy Research 2003; 17: 713-716.

Fennell CW, Lindsey KL, McGaw LJ. Sparg SG, Stafford GI, Elgorashi EE, Grace OM, van Staden J. Assessing African medicinal plants for efficacy and safety: pharmacological screening and toxicity. Journal of Ethnopharmacology 2004; 94: 205-217.

Ferraz AC, Angelucci MEM, Da Costa ML, Batista IR, De Oliveira BH, Da Cunha C. Pharmacological evaluation of ricinine, a central nervous system stimulant isolated from *Ricinus communis*. Pharmacology Biochemistry and Behaviour 1999; 63: 367-375.

Finar IL. 1975. Organic chemistry Volume 2: Stereochemistry and the chemistry of natural products 5th edition. Longman Singapore Publisher Pte Ltd. Singapore.

Fivelman QL, Walden JC, Sithe PJ, Folb PI, Barnes KI. The effect of artesunate combined with standard antimalarials against chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum in vitro*. Transactions of the Royal Society of Tropical Medicine and Hygiene 1999; 93: 429-432.

Fouche G, Cragg GM, Pillay P, Kolesnikova N. *In vitro* anticancer screening of South African plants. Journal of Ethnopharmacology 2008; 119: 455-461.

Foukaridis GN, Muntingh GL, Osuch E. Application of diode array detection for the identification of poisoning by traditional medicines. Journal of Ethnopharmacology 1994; 41: 135-146.

Fu P. Robinson R. Isolation and Characterisation of pyridinium oxidase B. Phytochemistry 1972; 11: 95-103.

Furst DE, Ulrich RW. 2007. Nonsteroidal anti-inflammatory drugs, disease-modifying antirheumatic drugs, nonopiod analgesics, and drugs used in gout, In: Katzung BG (Ed), Basic and Clinical Pharmacology 10th ed. McGraw-Hill Companies, Inc. United States of America.

Fyhrquist P, Mwasumbi L, Vuorela P, Vuorela H, Hiltunen R, Murphy C, Adlercreutz H. Preliminary antiproliferative effects of some species of *Terminalia*, *Combretum* and *Pteleopsis* collected in Tanzania on some human cancer cell lines. Fitoterapia 2006; 77: 358-366.

Gathirwa JW, Rukunga GM, Njagi ENM, Omar SA, Mwitari PG, Guantai AN, Tolo FM, Kimani CW, Muthaura CN, Kirira PG, Ndunda TN, Amalemba G, Mungai GM, Ndiege IO. The *in vitro* anti-plasmodial and *in vivo* anti-malarial efficacy of combinations of some medicinal plants used traditionally for treatment of malaria by the Mery community in Kenya. Journal of Ethnopharmacology 2008; 115: 223-231.

Germanò MP, Angelo VD, Biasini T, Sanogo R, De Pasquale R Catania S. Evaluation of the anti-oxidant properties and bioavailability of free and bound phenolic acids from *Trichilia emetica* Vahl. Journal of Ethnopharmacology 2006; 105: 368-73.

Gessler MC, Nkunya MHH, Mwasumbi LB, Heinrich M, Tanner M. Screening Tanzanian medicinal plants for antimalarial activity. Acta Tropica 1994; 56: 66-77.

Geyid A, Abebe D, Dabella A, Zewdneh M, Aberra F, Teka F, Kebede T, Urga K, Yersaw K, Biza T, Mariam BH, Guta M. Screening of some medicinal plants of Ethiopia for their anti-microbial properties and chemical profiles. Journal of Ethnopharmacology 2005; 97: 421-27.

Ghayur MN, Gilani AH, Khan A, Amor EC, Villaseňor IM, Choudhary MI. Presence of calcium antagonist activity explains the use of *Syzygium samarangense* in diarrhoea. Phytotherapy Research 2006; 20: 49-52.

Gilani AH, Rahman A. Trends in ethnopharmacology. Journal of Ethnopharmacology 2005; 100: 43-49.

Gort E. Changing traditional medicine in rural Swaziland: the effects of the global system. Social Science & Medicine. 1989; 29: 1099-1104.

Green E and Makhubu L. Traditional healers in Swaziland: Toward improved cooperation between the traditional and modern health sectors. Social Science and Medicine 1984; 18:1071-1078.

Grice HC. Safety evaluation of butylated hydroxyanisole from the perspective of effects on forestomach and oesophageal squamous epithelium. Food Chemistry and Toxicology 1988; 24: 717-728.

Gülçin I, Berashvili D, Gepdiremen A. Antiradical and antioxidant activity of total anthocyanins from *Perilla pankinesis* decne. Journal of Ethnopharmacology 2005; 101: 287-293.

Gülçin I, Küfrevioğlu I, Oktay M, Büyükokuroğlu ME. Antioxidant, anitmicrobial and antiulcer and analgesic activities of nettle (*Urtica dioica* L.). Journal of Ethnopharmacology 2004; 90: 205-215.

Gunatilaka AAL, Sirimane SR, Sotheeswaran S, Sriyani HTB. Flavonoids of *Gardenia cramerii* and *G. fosbergii* bud exudates. Phytochemistry 1982; 21: 805-806.

Gupta S, Thapar MM, Wernsdorfer WH, Bjorkman A. *In vitro* interaction of artemisinin with atovaquone quinine and mefloquine against *Plasmodium falciparum*. Antimicrobial Agents Chemotherapy 2002; 46: 1510-1515.

Gutiérrez SP, Sánchez MAZ, González CP, García LA. Antidiarrhoeal activity of different plants used in traditional medicine. African Journal of Biotechnology 2007; 6: 2988-2994.

Halliwell B and Gutteridge JMC. 1989. Free radicals in Biology and Medicine, 2nd Ed. Oxford University Press, New York.

Halliwell B. Free radicals, antioxidants, and human disease: curiosity, cause or consequence? Lancet 1994; 344: 721-724.

Hamburger M and Hostettmann K. Analytical aspects of drugs of natural origin. Journal of Pharmaceutical and Biomedical Analysis 1989; 7: 1337-1349.

Hamburger M, Hostettmann K. Bioactivity in plants: the link between phytochemistry and medicine. Phytochemistry 1991; 30: 3864-3874.

Hammer KA, Carson CF, Riley TV. Antimicrobial activity of essential oils and other plant extracts. Journal of Applied Microbiology 1999; 86: 985-990.

Hastings IM. Watkins WM. Tolerance is the key to understanding antimalarial drug resistance. Trends in Parasitology 2006; 22: 71-77.

Hatano T, Edamatsu R, Haramatsu M, Mori A, Fujita Y, Yasuhara E, Yashida T and Okuda T. Effects of the interaction of tannins with co-existing substances. VI [1)] Effects of tannins and related polyphenols on superoxide anion radical and on 1,1-diphenyl-2-picrylhydrazil radical. Chemical and Pharmaceutical Bulletin 1989; 37: 2016-2021.

Havsteen B. Flavonoids, a class of natural products of high pharmacological potency. Biochemical Pharmacology 1983; 32: 1141-1148.

Hawker LE and Linton AH. 1971. Micro-organisms function, form and environment. Edward Arnold (Publishers) Ltd.

Heinrich M, Barnes J, Gibbons S, Williamson EM. 2004. Fundamentals of Pharmacognosy and Phytotherapy. Churchill Livingstone Elsevier Limited Spain.

Hentze MW, Muckenthaler MU, Andrews NC. Balancing acts: molecular control of mammalian iron metabolism. Cell 2004: 117; 285-297.

Herbert RB, Moody CJ. Moraceae: Alkaloids of *Ficus septica*. Phytochemistry 1972; 11: 1184.

Hong YN. Detection of a pteridine oxidase in plants. Plant Science Letters 1980; 18: 169-175.

Hořejší V. Separation of *Ricinus communis* lectins by affinity chromatography. Journal of Chromatography 1979; 169: 457-458.

Horie T, Kawamura Y, Yamamoto H, Kitou T, Yamashita K. Synthesis of 5,8-dihydroxy-6,7-dimethoxyflavones and revised structures for some natural flavones. Phytochemistry 1995; 39: 1201-1210.

Horn JR. 2007. Appendix II: Important drug interactions & their mechanisms. In: Katzung BG: Basic & Clinical Pharmacology, 10th edition. McGraw-Hill Companies, Inc. United States of America.

Hou WC, Lin RD, Cheng KT, Hung YT, Cho CH, Chen CH, Hwang SY, Lee MH. Free radical-scavenging activity of Taiwanese native plants. Phytomedicine 2003; 10: 170-175.

Humphreys H. 2007. *Staphylococcus* skin infections; osteomyelitis; food poisoning; foreign body infections; MRSA, In: Greenwood D, Slack R, Peutherer J, Barer M. (Eds) Medical Microbiology, 7th edition. Churchill Livingstone, Elsevier.

Ibezim EC, Esimone CO, Nnamani PO, Onyishi IV, Brown SA, Obodo CE. *In vitro* study of the interaction between some fluoroquinolones and extracts of *Kola nitida* seed. African Journal of Biotechnology 2006; 5: 1781-1784.

Ilavarasan R, Mallika M, Venkataraman S. Anti-inflammatory and free radical scavenging activity of *Ricinus communis* root extract. Journal of Ethnopharmacology 2006; 103: 478-480.

Inglis TJJ. 1996. Microbiology and Infection. A clinically-oriented core text with self-assessment. Churchill Livingstone. Longman Singapore Publishers (Pte) Ltd.

Inouye H, Takeda Y, Nishimura H. Two new iridoid glucosides from *Gardenia jasminoides* fruits. Phytochemistry 1974; 13: 2219-2224.

Iriyama K, YoshiuraM, Asai H. Visible absorption spectra of chlorophyll b in methanol, dioxane and/or water solutions. Chemistry Letters 1974: 997-1000.

Iwu MM. 1993. Handbook of African Medicinal Plants. C.R.C. Press, Florida.

Iwu MM. 1994. African medicinal plants in the search for new drugs base on ethnobotanical leads. In: Chadwick DJ, Marsh J (Eds). Ethnobotany and the Search for New Drugs. Ciba Foundation Symposium, vol. 185 John Wiley and Sons, Chichester.

Jakupovic J, Grenz M, Bohlmann F, Mungai Gm. 12β-Hydroxyabieta-7,13-diene and other constituents from East African *Helichrysum* species. Phytochemistry1990; 29: 1589-1590.

Jensen B, Trager W. *Plasmodium falciparum* in culture: Use of outdated erythrocytes and description of the candle jar method. Journal of Parasitology 1977; 63: 883-886.

Joshi KC, Sharma T. Triterpenoids and some other constituents from *Dichrostachys cinerea*. Phytochemistry 1974; 13: 2010-2011.

Jourbert E, Gelderblom WCA, Louw A, de Beer D. South African herbal teas: *Aspalathus linearis*, *Cyclopia* spp. and *Athrixia phyllicoides* – A review. Journal of Ethnopharmacology 2008; 119: 376- 412.

Kähkönen MP, Hopia AI, Vuorela HJ, Rauha JP, Pihlaja K, Kujala TS, Heiononen M. Antioxidant activity of plant extracts containing phenolic compounds. Journal of Agriculture and Food Chemistry 1999; 47: 3954-3962.

Kaido TL, Veale DJH, Havlik I, Rama DBK. Preliminary screening of plants used in South Africa as traditional herbal remedies during pregnancy. Journal of Ethnopharmacology 1997; 55: 185-191.

KaiScience 2007. Dennis Kunkel Microscopy, Inc.
www.kaiscience.com/thumbimage.php?id=156 , accessed on the 15th April 2009.

Kamatou GPP, van Zyl RL, van Vuuren SF, Figueiredo AC, Barroso JG, Pedro LG, Viljoen AM. Seasonal variation in essential oil composition, oil toxicity and the biological activity of solvent extracts of three South African *Salvia* species. South African Journal of Botany 2008; 74: 230-237.

Kamatou GPP, Viljoen AM, Figueiredo AC, Tilney PM, van Zyl RL, Barroso JG , Pedro LG, van Vuuren SF. Trichomes, essential oil composition and biological activities of *Salvia albicaulis* Benth. and *S. dolomitica* Codd, two species from the Cape region of South Africa. South African Journal of Botany 2007; 73: 102-108.

Kamatou GPP, Viljoen AM, Ono-Bwalya AB, van Zyl RL, van Vuuren SF, Lourens ACU, Baser KHC, Demicri B. Lindsey KL, van Staden J, Steenkamp P. The *in vitro* pharmacological activities and chemical investigation of three South African *Salvia* species, Journal of Ethnopharmacology 2005; 102: 382-390.

Kamatou GPP, Viljoen AM, van Vuuren SF, van Zyl RL. *In vitro* evidence of antimicrobial synergy between *Salvia chamelaeagnea* and *Leonotis leonurus*. South African Journal of Botany 2006; 72: 634-636.

Kamatou GPP. Pharmacological and photochemistry of selected South African *Salvia* species 2006 PhD. University of the Witwatersrand, Johannesburg, South Africa.

Kambizi L, Afolayan AJ. An ethnobotanical study of plants used for the treatment of sexually transmitted diseases (*njovhera*) in Guruve District, Zimbabwe. Journal of Ethnopharmacology 2001; 77: 5-9.

Kaur AD, Ravichandran V, Jain PK, Agrawal RK. High-performance thin layer chromatography method for estimation of conessine in herbal extract and pharmaceutical dosage formulations. Journal of Pharmaceutical and Biomedical Analysis 2008; 46: 391-394.

Kayser FH. 2005. Bacteria as human pathogens. In Kayser FH, Bienz KA, Eckert J, Zinkernagel RM (Ed). Medical Microbiology. Georg Thieme Verlag, New York.

Khan MR, Ndaalio G, Nkunya MHW, Wevers H. Studies on the rationale of African traditional medicine. Part II – preliminary screening of medicinal plants for anti-gonococci activity. Pakistan Journal of Science Research 1978; 27: 189-192.

King A and Melton DW. Characterisation of cDNA clones for hypoxanthine-quanine phosphoribosyltransferase from the human malarial parasite, *Plasmodium falciparum*: comparisons to the mammalian gene and protein. Nucleic Acids Research 1987; 15: 10469-10481.

Kiyishe T. Toyoshima S, Osawa T. Effect of *Ricinus communis* lectins on the membrane fluidity of human peripheral lymphocytes. Biochemical and Biophysical Research Communications 1974; 60: 681-686.

Klaassen CD, 2001. Principles of toxicology and treatment of poisoning, In: Hardman JG, Limbird LE (Eds), Gilman AG. Goodman and Gilman's The Pharmacological Basis of Therapeutics 10th ed. McGraw-Hill Companies, Inc. United States of America.

Kohen R and Nyska A. Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. Toxicologic Pathology 2002; 30: 620-650.

Koné WM, Atindehou KK, Terreaux C, Hostettman K, Traoré D, Dosso M. Traditional medicine in North Cote-d'Ivoire: screening of 50 medicinal plants for antibacterial activity. Journal of Ethnorpharmacology 2004; 93: 43-49.

Lall N, Meyer JJM. Antibacterial activity of water and acetone extracts of roots of *Euclea natalensis*. Journal of Ethnopharmacology 2000; 72: 313-316.

Lalli JYY, van Zyl RL, van Vuuren SF, Viljoen AM. *In vitro* biological activities of South African *Pelargonium* (Geraniaceae) species. South African Journal of Botany 2008; 74: 153-157.

Lampiris HW, Maddix DS. 2007. Clinical use of antimicrobial agents. In: Katzung BG. Basic and Clinical Pharmacology, 10th edition. McGraw-Hill Companies, Inc. United States of America.

Leong CAN, Tako M, Hanashiro I, Tamaki H. Antioxidant flavonoid glycosides from the leaves of *Ficus pumila* L. Food Chemistry 2008; 109: 415-420.

Li X, Xiong Z, Ying X, Cui L, Zhu W, Li F. A rapid ultra-performance liquid chromatography-electrospray ionization tandem mass spectrometric method for the qualitative and quantitative analysis of the constituents of the flower of *Trollius ledibouri* Reichb. Analytica Chimica Acta 2006; 580: 170-180.

Li YC, Kuo YH. A monoterpenoid and two simple phenols from heartwood of *Ficus microcarpa*. Phytochemistry 1998; 49: 2417-2419.

Liu Y, Abreu P. Tirucallane triterpenes from the roots of *Ozoroa insignis*. Phytochemistry 2006; 67: 1309-1315.

Lombard S, Helmy ME, PiéroniG. Lipolytic activity of ricin from *Ricinus sanguineus* and *Ricinus communis* on neutral lipids. Biochemistry Journal 2001; 358: 773-781.

Lopes D. Villela CT, Kaplan MAC, Carauta JPP. Moretenolactone, a β-lactone hopanoid from *Ficus insipida.* Phytochemistry 1993; 34: 279-280.

Lourens ACU, Reddy D, Başer KHC, Viljoen AM, Van Vuuren SF. *In vitro* biological acitivity and essential oil composition of four indigenous South African *Helichrysum* species. Journal of Ethnopharmacology 2004; 95: 253-258.

Lourens ACU, Viljoen AM, van Heerden FR. South African *Helichrysum* species: A review of the traditional uses, biological activity and phytochemistry. Journal of Ethnopharmacology 2008; 119: 630-652.

Lugnier AAJ, Creppy EE, Le Meur MA, Gerlinger P, Dirheimer G. Reciprocal interactions of ricin from *Ricinus communis* L. seeds with eukaryotic ribosomes. FEBS Letters 1977; 76: 166-172.

Mabeza GF, Loyevsky M, Gordeuk VR, Weiss G. Iron chelation therapy for malaria: a review. Pharmacology and Therapeutics 1999; 81: 53-75.

MacKinnon S. Durst T, Arnason JT. Antimalarial activity of tropical Meliacea extracts and gedunin derivatives. Journal of Natural Products 1997; 60: 336-341.

Magassouba FB, Diallo A, Kouyat´e M, Mara F, Mara O, Bangoura O, Camara A, Traor´e S, Diallo AK, Zaoro M, Lamah K, Diallo S, Camara G, Traor´e S, K´eita A, Camara MK, Barry R, K´eita S, Oular´e K, Barry MS, Donzo M, Camara K, Tot´e K, Vanden Berghe D, Tott´e J, Pieters L, Vlietinck AJ, Bald´e AM. Ethnobotanical survey and antibacterial activity of some plants used in Guinean traditional medicine. Journal of Ethnopharmacology 2007; 114: 44-53.

Mahasneh AM. Screening of some indigenous Qatari medicinal plants for antimicrobial activity. Phytotherapy Research 2002; 16: 751-753.

Makkar HPS, Siddhuraju P, Becker K (Eds). 2007. Methods in Molecular Biology, Vol 393: Plant secondary metabolites. Human Press Inc., Totowa, NJ.

Malagon F and Castillo OG. On the labeling of malaria parasites *in vitro* with ^{3}H-hypoxanthine while developing an infection in the mouse. Acta Protozoologica 2000; 39: 281-288.

Manna P, Sinha M, Pal P, Sil PC. Arjunolic acid, a triterpenoid saponin, ameliorates arsenic-induced cyto-toxicity in hepatocytes. Chemico-Biological Interactions 2007; 170: 187-200.

Marcos LA, DuPont HL. Advances in defining etiology and new therapeutic approaches in acute diarrhoea. Journal of Infection 2007; 55: 385-393.

Marston A, Hostettmann K. 1999. Biological and chemical evaluation of plant extracts and subsequent isolation strategy, In: Bohlin L, Bruhn JG (Eds). Bioassay methods in natural product research and drug development. Kluwer Academic Publishers, USA.

Masoko P. Picard J, Eloff JN. Antifungal activitites of six South African *Terminalia* species (Combretaceae). Journal of Ethnopharmacology 2005; 99: 301-308.

Mathabe MC, Nikolova RV, Lall N, Nyazema NZ. Antibacterial activities of medicinal plants used for the treatment of diarrhea in Limpompo Province, South Africa. Journal of Ethnopharmacology 2006; 105: 286-293.

Mau JL, Lin HC, Song SF. Antioxidant properties of several speciality mushrooms. Food Research International 2002; 35: 519-526.

Mbatchi, SF, Mbatchi B, Banzouzi JT, Bansimba T, Nsonde Ntandou GF, Oumba JM, Berry A, Benoit-Vical F. *In vitro* antiplasmodial activity of 18 plants used in Congo Brazzavile traditional medicine. Journal of Ethnopharmacology 2006; 104: 168-174.

McCune LM, Johns T. Antioxidant activity in medicinal plants associated with the symptoms of diabetes mellitus used by the Indigenous Peoples of the North American boreal forest. Journal of Ethnopharmacology 2002; 82: 197-205.

McGaw LJ, Eloff JN. Ethnoveterinary use of southern African plants and scientific evaluation of their medicinal properties. Journal of Ethnopharmacology 2008; 119: 559-574.

McGaw LJ, Lall N, Meyer JJM, Eloff JN. The potential of South African plants against *Mycobacterium* infections. Journal of Ethnopharmacology 2008; 119: 482-500.

Medical Microbiology. 2007. http://micro.digitalproteus.com/morphology2.php, accessed on the 24th July 2009.

Miliauskas G, Venskutonis PR, van Beek TA. Screening of radical scavenging activity of some medicinal and aromatic plant extracts. Food Chemistry 2004; 85: 231-237.

Mølgaard P, Nielsen SB, Rasmussen DE, Drummond RB, Makaza N, Andreasse J. Antihelmintic screening of Zimbabwean plants traditionally used against schistosomiasis. Journal of Ethnopharmacology 2001; 74: 257-264.

Molyneux P. The use of DPPH to estimate antioxidant activity. Songklanakarin Journal of Science and Technology 2004; 26: 211-219.

Moreno ACR, Filho AF, Gomes TAT, Ramos STS, Montermor LPG, Tavares VC, Filho LS, Irino K, Martinez MB. Etiology of childhood diarrhoea in the northeast of Brazil: significant emergent diarrhoeal pathogens. Diagnostic Microbiology and Infectious Diseases 2008. dio:10.1016/j.diagmicrobio.2008.03.017.

Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. Journal of Immunological Methods 1983; 65: 55-63.

Mukherjee PK, Saha K, Murugesan T, Mandal SC, Pal M, Saha BP. Screening of anti-diarrhoeal profile of some plant extracts of a specific region of West Bengal, India. Journal of Ethnopharmacology 1998; 60: 85-89.

Mukinda JT, Syce JA. Acute and chronic toxicity of aqueous extract of *Artemisia afra* in rodents. Journal of Ethnopharmacology 2007; 112: 138-144.

Muregi FW, Ishih A, Miyase T, Suzuki T, Kino H, Amano T, Mkoji GM, Terada M. Antimalarial activity of methanolic extracts from plants used in Kenyan ethnomedicine and

their interactions with chloroquine (CQ) against a CQ-tolerant rodent parasite, in mice. Journal of Ethnopharmacology 2007; 111: 190-195.

Mustofa AV, Benoit-Vical F, Pélissier Y, Koné-Bamba D, Mallié M. Antiplasmodial activity of plant extracts used in west African traditional medicine. Journal of Ethnopharmacology 2000; 73: 145-151.

Nakamura Y, Kawakami M, Yoshihiro A, Miyoshi N, Ohigashi H, Kawai K, Osawa T, Uchida K. Involvement of the mitochondrial death pathway in chemopreventive benzyl isothiocyanate-induced apoptosis. Journal of Biological Chemistry 2002; 277: 8492–8499.

Nakatani M Iwashita T, Naoki H, Hase T. Structure of a limonoid antifeedant from *Trichilia roka*. Phytochemistry 1985; 24: 195-196.

Nascimento M, ArrudaA.C, Arruda MSP, Müler AH, Yoshioka CY. Aripuanin, a megastigmane skeleton from *Ficus aripuanensis.* Phytochemical communication, Fitoterapia 1999; 70: 628-629.

Nataro JP, Kaper JB. Diarrheagenic *Escherichia coli*. Clinical Microbiology 1998; 11: 142-201.

National Department of Health 2008a. Guidelines for the prevention of malaria in South Africa. www.doh.gov.za, accessed on the 11th August 2008.

National Department of Health 2008b. Guidelines for the treatment of malaria in South Africa. www.doh.gov.za, accessed on the 11th August 2008.

National Malaria Control Program Annual Report 2004/05. Ministry of Health and Social Welfare. Swaziland.

Ndhlala AR, Finnie JF, van Staden J. Antibacterial, antifungal and anti-inflammatory effects of medicinal concoctions used in South Africa. South African Journal of Botany 2008; 74: 374.

Neuwinger HD. 1996. African Ethnobotany Poisons and Drugs. Chapman and Hall, Weinheim.

Ngouammegne ET, Fongang SR, Ngouela S, Boyom FF, Rohmer M, Tsamo E, Gut J, Rosenthal PJ. Endodesmiadol, a friedelane triterpenoid, and other antiplasmodial compounds from *Endodesmia calophylloides*. Chemical Pharmacology Bulletin 2008; 56: 374-377.

Nishizawa M, Yamada H, Sano J, Itô, S, Hayashi Y, Ikeda K, Chairul, Shiro M, Tokuda H. Structure of syzygiol: a skin-tumor promotion inhibitor. Tetrahedron Letters 1991; 32: 211-212.

Nordberg J, Arnèr ESJ. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. Free Radical Biology & Medicine 2001; 31: 1287 – 1312.

Northwestern University, Feinberg School of Medicine**,** Haldar Lab. 2004. *Plasmodium falciparum* www.haldarlab.northwestern.edu/pagepic/pf-life.jpg, accessed on the 7th November 2007.

Okpekon T, Yolou S, Gleye C, Roblot F, Loiseau P, Bories C, Grellier P, Frappier F, Laurens A, Hocquemiller R. Antiparasitic acitivities of medicinal plants used in Ivory Coast. Journal of Ethnopharmacology 2004; 90: 91-97.

Oktay M, Gülçin I, Küfrevioğlu ÖI. Determination of *in vitro* antioxidant activity of fennel (*Foeniculum vulgare*) seed exracts. Lebensmittel-Wissenchaft und Technologie 2003; 36: 263-271.

Okusa PN, Penge O, Debleeschouwer M, Duez P. Direct and indirect antimicrobial effects and antioxidant activity of *Cordia gilletii* De Wild (Boraginaceae). Journal of Ethnopharmacology 2007; 112: 476-481.

Olliaro P. Mode of action and mechanisms of resistance for antimalarial drugs. Pharmacology and Therapeutics 2001; 89: 207-219.

Omulokoli E, Khan B, Chhabra SC. Antiplasmodial activity of four Kenyan medicinal plants. Journal of Ethnopharmacology 1997; 56: 133-137.

Outtara Y, Sanon S, Traore Y, Mahiou V, Azas N, Sawadogo L. Antimalarial activity of *Swartzia madagascarensis* Desv. (Leguminosae), *Combretum glutinosum* Guill. and Perr. (Combretaceae) and *Tinospora bakis* Miers. (Menispermaceae), Burkina Faso medicinal plants. African Journal of Traditional CAM 2006; 3: 75-81.

Ozaki A, Kitano M, Furusawa N, Yamaguchi H, Kuroda K, Endo G. Genotoxicity of *Gardenia* yellow and its components. Food and Chemical Toxicology 2002; 40: 1603-1610.

Palombo EA. 2006. Traditional plant and herbal remedies used in the treatment of diarrheal disease: mode of action, quality, efficacy, and safety considerations, In: Ahmed I, Aqil F, Owais M (Eds). Modern Phytomedicine. Turning Medicinal Plants into Drugs. Wiley-VCH Verlag GmbH & Co. KGaA, Weiheim.

Pelczar Jr. MJ, Chan ECS, Krieg NR. 1986. Microbiology, 5th edition. McGraw-Hills Inc. United States of America.

Phillips DAM. 2007, Visuals Unlimited. www.humanillnesses.com/original/U-Z/Yeast-Infection-Vaginal.html, accessed on the 30th March 2009.

Pietta P, Mauri P, Gardana C, Maffei Facino R, Carini M. High-performance liquid chromatographic determination of flavonoid glucosides from *Helichrysum iItalicurn*. Journal of Chromatography 1991; 537: 449-452.

Pillay P, Maharaj VJ, Smith PJ. Investigating South African plants as a source of new antimalarial drugs. Journal of Ethnopharmacology 2008; 119: 438-454.

Pink R, Hudson A, Mouries MA, Bendig M. Opportunities and challenges in antiparasitic drug discovery. Nature Reviews Drug Discovery 2005; 4: 727-740.

Pistelli L, Chiellini EE, Morelli I. Flavonoids from *Ficus pumila*. Biochemical Systematics and Ecology 2000; 28: 287-289.

Plaa GL. 2007. Introdution to toxicology: Occupational & Environmental, In: Katzung BG (Ed), Basic and Clinical Pharmacology 10th ed. McGraw-Hill Companies, Inc. United States of America.

Popat A, Shear NH, Malkiewicz I, Stewart MJ, Steenkamp V, Thomson S, Neuman MG. The toxicity of *Callilepis laureola*, a South African traditional herbal medicine. Clinical Biochemistry 2001; 34: 229-236.

Prère MF, Bacrie SC, Baron O, Fayet O. Bacterial aetiology of diarrhoea in young children: high prevalence of enteropathogenic *Escherichia coli* (EPEC) not belonging to the classical EPEC serogroups. Pathologie Biologie 2006; 54: 600-602.

Prinsloo G, Meyer JJM. *In vitro* production of phytoalexins by *Helichrysum kraussii*. South African Journal of Botany 2006; 72: 482-483.

Prozesky EA, Meyer JJM, Louw AI. *In vitro* antiplasmodial activity and cytotoxicity of ethnobotanically selected South African plants. Journal of Ethnopharmacology 2001: 76: 239-245.

Qian GS, Wang Q, Leung KSY, Qin Y, Zhao Z, Jiang ZH. Quality assessment of Rhizoma et *Radix Notopterygii* by HPTLC and HPLC fingerprinting and HPLC quantitative analysis. Journal of Pharmaceutical and Biomedical Analysis 2007; 44: 812-817.

Quettier-Deleu C, Gressier B, Vasseur J, Dine T, Brunet C, Luyckx M, Cazin M, Cazin J, Bailleul F, Trotin F. Phenolic compounds and antioxidant activities of buckwheat (*Fagopyrum esculentum* Moench) hulls and flour. Journal of Ethnopharmacology 2000; 72: 35-42.

Rao RJ, Tiwari AK, Kumar US, Reddy SV, Ali AZ, Rao JM. Novel 3-*O*-Acyl Mesquitol Analogues as free-radical scavengers and enzyme inhibitors: synthesis, biological evaluation and structure-activity relationship. Bioorganic & Medicinal Chemistry Letters 2003; 13: 2777-2780.

Rasoanaivo P. Ratsimamanga-Urverg S, Amanitrahasimbola D, Rafrato H, Rokoto-R A. Criblige d'extraits de plantes de Madagascar pour recherché d'activité antipaludique et d' effet pptentializateur de la chloroquinine. Journal of Ethnopharmacology 1999; 64: 117-126.

Rauf A, Shah AH, Kamran MA. Phytochemical screening of some plants used in traditional medicine. Journal of Pure Applied Science 1986; 5: 19-23.

Rautenbach M, Vlok Nm, Stander M, Hoppe HC. Inhibition of malaria parasite blood stages by tyrocidines, membrane-active cyclic peptide antibiotics from *Bacillus brevis*. Biochemica et Biophysica Acta 2007; 1768: 1488-1497.

Rea AI, Schmidt JM, Setzer WN, Sibanda S, Taylor C, Gwebu ET. Cytotoxicity of *Ozoroa insignis* from Zimbabwe. Fitoterapia 2003; 74: 732-735.

Reddy GCS, Ayengar KNN, Rangaswami S. Triterpenoids of *Gardenia latifolia*. Phytochemistry 1975; 14: 307.

Reid KA, Maes J, Maes A, van Staden J, De Kimpe N, Mulholland DA, Verschaeve L. Evaluation of the mutagenic and antimutagenic effects of South African plants. Journal of Ethnopharmacology 2006; 106: 44-50.

Reutrakul V, Krachangchaeng C, Tuchinda P, Pohmakotr M, Jaipetch T, Yoosook C, Kasisit J, Sophasan S, Sujarit K, Santisuke T. Cytotoxic and anti-HIV-1 constituents from leaves and twigs of *Gardenia tubifera*. Tetrahedron 2004; 60: 1517-1523.

Rice-Evans CA, Miller NM, Paganda G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radical Biology and Medicine 1996; 20: 933-956.

Roberts LM, Lamb FI, Pappin DJC, Lord JM. The primary sequence of *Ricinus communis* agglutinin. Comparison with ricin. The Journal of Biological Chemistry 1985; 260: 15682-15686.

Rosa A, Deiana M, Atzeri A, Corona G, Incani A, Melis MP, Appendino G, Dess MA. Evaluation of the antioxidant and cytotoxic activity of arzanol, a prenylated α-pyrone-phloroglucinol etherodimer from *Helichrysum italicum* subsp. *microphyllum*. Chemico-Biological Interactions 2007; 165: 117-126.

Rosenthal PJ. 2006. Antiprotozoal drugs, In: Katzung BG (Ed). Basic and Clinical pharmacology 10th edition. McGraw Hill Professional.

Rukuchaisirikul V, Naovanit Sa, Taylor WC, Bubb WA, Dampawan P. A sesquiterpene from *Gardenia sootepensis*. Phytochemistry 1998; 48: 197-200.

Runyoro DKB, Matee MIN, Ngassapa OD, Joseph CC, Mbwambo ZH. Screening of Tanzania medicinal plants for anti-*Candida* activity. BMC Complementary and Alternative Medicine 2006; 6: 11. doi: 10.1186/1472-6882-6-11.

Ruxin J, Paluzzi JE, Wilson PA, Tozan Y, Kruk M, Teklehaimanot A. Emerging consensus in HIV/AIDS, malaria, tuberculosis and access to essential medicines. Lancet 2005; 365: 618-621.

Saeed MA, Sabir AW. Irritant potential of triterpenoids from *Ficus carica* leaves. Fitoterapia 2002; 73: 417-420.

Saha NC, Kaviraj A. Acute and chronic toxicity of tannic acid and spent bark of cinchona to tilapia *Oreochromis mossambicus*. Aquaculture 1996; 145: 119-127.

Sala A, Recio M C, Schinella GR, Máñez S, Giner RM, Cerdá-Nicolás M, Ríos JL. Assessment of the anti-inflammatory activity and free radical scavenger activity of tiliroside. European Journal of Pharmacology 2003; 461: 53-61.

Salisbury FB, Ross CW. 1992. Plant Physiology 4th ed. Wadsworth Publishing Company, California.

Salle AJ. 1973. Fundamental Principles of Bacteriology, 7th edition. McGraw-Hill Inc. United Kingdom.

Samie A, Obi CL, Bessong PO, Namrita L. Activity profiles of fourteen selected medicinal plants from Rural Venda communities in South Africa against fifteen clinical bacterial species. African Journal of Biotechnology 2005: 4; 1443-1451.

Samuelsson G. 1999. Drugs of natural origin. A textbook of pharmacognosy. Swedish Pharmaceutical Society. Swedish Pharmaceutical Press, Stockholm.

Sandabe UK, Onyeyili PA, Chibuzo GA. Phytochemical screening and effect of aqueous extract of *Ficus sycomorus* L. (Moraceae) stembark on muscular activity in laboratory animals. Journal of Ethnopharmacology 2006; 104: 283-285.

Sanon S. Ollivier E, Azas N, Mahiou V, Gasquet M, Outtara CT, Nebie I, Traore AS, Esposito F, Balansard G, Timon-David P, Fumoux F. Ethnobotanical survey and *in vitro* antiplasmodial activity of plants used in traditional medicine in Burkina Faso. Journal of Ethnopharmacology 2003; 86: 143-147.

Sarradin P, Le Bris N, Le Gall C, Rodier P. Fe analysis by the ferrozine method: Adaptation to FIA towards *in situ* analysis in hydrothermal environment. Talanta 2005; 66: 1131-1138.

Saxena M, Faridi U, Mishra R, Gupta MM, Darokar MP, Srivastava SK, Singh D, Luqman S, Khanuja SP. Cytotoxic agents from *Terminalia arjuna*. Planta Medica 2007; 73: 1486-1490.

Scharf Photo.com. 2009.
http://www.scharfphoto.com/fine_art_prints/archives/000608.php, accessed on the 15th April 2009.

Schwikkard S, van Heerden F. Antimalarial activity of plant metabolites. Natural Products Report 2002; 19: 1-19.

Science Hypermedia. 1996. High performance liquid chromatography (HPLC).
http://elchem.kaist.ac.kr/vt/chem-ed/sep/lc/hplc.htm, accessed 19th February 2008.

Senevirathne M, Kim S, Siriwardhana N, Ha J, Lee K, Jeon Y. Antioxidant potential of *Ecklonia cava* on reactive oxygen species scavenging, metal chelating, reducing power and lipid peroxidation inhibition. Food Science and Technology International 2006; 12: 27-38.

Shahidi F, Janitha PK, Wnasundara PD. Phenolic antioxidants. Critical Review in Food Sciences and Nutrition 1992; 32: 67-103.

Shai LJ, McGaw LJ, Masoko P, Eloff JN. Antifungal and antibacterial activity of seven traditionally used South African plant species active against *Candia albicans*. South African Journal of Botany 2008; 74: 677-684.

Sharma P, Sharma JD. *In vitro* hemolysis of human erythrocytes – by plant extracts with antiplasmodial activity. Journal of Ethnopharmacology, 2001; 74: 239-243.

Sherwin FR. 1990. Antioxidants. In: Branen R, (Ed), Food Additives. Marcel Dekker, New York.

Silva GL, Gil RR, Cui B, Chai H, Santisuk Ti, Srisook E, Reutrakul V, Tuchinda P, Sophasan S, Sujarit S, Upatharq S, Lynn SM, Farthing JE, Yang SL, Lewis JA, O'Neill MJ, Farnsworth NR, Cordell GA, Pezzutoand JM, Kinghom D. Novel cytotoxic ring-A seco-cycloartane triterpernes from *Gardenia coronaria* and *G. sootepensis*. Tetrahedron 1997; 53: 529-538.

Simo CCF, Kouam SF, Poumale HMP, Simo IK, Ngadjui BT, Green IR, Krohn K. Benjaminamide: A new ceramide and other compounds from the twigs of *Ficus benjamina* (Moraceae). Biochemical Systematics and Ecology 2008; 36: 238-243.

Sitton D, West CA. Casbene: An anti-fungal diterpene produced in cell-fee extracts of *Ricinus communis* seedlings. Phytochemistry 1975; 14: 1921-1925.

Skalli S, Zaid A, Soulaymani R. Drug interactions with herbal medicines. Therapeutic Drug Monitoring 2007; 29: 679-686.

Škerget M, Kotnik P, Hadolin M, Hraš AR, Simonič M, Knez Z. Phenols, proanthocyanidins, flavones and flavonols in some plant materials and their antioxidant activities. Food Chemistry 2005; 89: 191-198.

Slinkard K, Singleton VL. Total phenol analyses: automation and comparison with manual methods. American Journal of Ecology and Viticulture 1977; 28: 49-55.

Snyman T, Stewart MJ, Grove A, Steenkamp V. Adulteration of South African Traditional Herbal Remedies. Therapeutic Drug Monitoring 2005; 27: 86-89.

Sofowora A. Recent trends in research into African medicinal plants. Journal of Ethnopharmacology 1993; 38: 209-214.

Sofowora A. Research on medicinal plants and traditional medicine in Africa. The Journal of Alternative and Complementary Medicine 1996; 2: 365-372.

Sowemimo AA, Fakoya FA, Awopetu I, Omobuwajo OR, Adesanya SA. Toxicity and mutagenic activity of some selected Nigerian plants. Journal of Ethnopharmacology 2007; 113: 427-432.

Sparg SG, van Staden J, Jäger AK. Efficiency of traditionally used South African plants against schistosomiasis. Journal of Ethnopharmacology 2000; 73: 209-214.

Srivastava AK, Srivastava SK, Syamsundar KV. Bud and leaf essential oil composition of *Syzygium aromaticum* from India and Madagascar. Flavour and Fragrance Journal 2005; 20: 51-53.

Srivastava SK, Srivastava SD, Chouksey BK. New constituents of *Terminalia alata*. Fitoterapia 1999; 70: 390-394.

Stadtman ER, Berlett BS. Reactive oxygen-mediated protein oxidation in aging and disease. Drug Metabolism Review 1998; 30: 225 -243.

Steele JC, Warhurst DC, Kirby GC, Simmonds MS. *In vitro* and *in vivo* evaluation of betulinic acid as an antimalarial. Phytotherapy Research 1999; 13: 115-119.

Steenkamp V, Fernandes AC, Van Rensburg CEJ. Screening of Venda medicinal plants for antifungal activity against *Candida albicans*. South African Journal of Botany 2007; 73: 256-258.

Steenkamp V, Mathivha E, Gouws MC, van Rensburg CEJ. Studies on antibacterial, antioxidant and fibroblast growth stimulation of wound healing remedies from South Africa. Journal of Ethnopharmacology 2004; 95: 353-357.

Steketee RW, Nahlen BL, Parise ME, Menendez C. The burden of malaria in pregnancy in malaria-endemic areas. American Journal of Tropical Medicine and Hygiene 2001; 64: 28-35.

Stern KR. 1997. Introductory Plant Biology 7th ed. WCB McGraw-Hill, United States of America.

Stewart MJ, Moar JJ, Steenkamp P, Kokot M. Findings in fatal cases of poisoning attributed to traditional remedies in South Africa. Forensic Science International 1999; 101: 177-183.

Storbeck KH, Kolar NW, Stander M, Swart AC, Prevoo D, Swart P. The development of an ultra performance liquid chromatography-coupled atmospheric pressure chemical ionization mass spectrometry assay for seven adrenal steroids. Analytical Biochemistry 2008; 372: 11-20.

Suksamrarn A, Suwunnapoch N, Aunchai M, Kuno M, Ratananukul P, Haritakun R, Jansakul C, Ruchirawat S. Ziziphine N/O/P and Q, new antiplasmodial cyclopeptide alkaloids from *Ziziphus oenoplia* var. *brunoniana*. Tetrahedron 2005; 61: 1175-1180.

Suksamrarn A, Tanachatchairatana T, Kanokmedhakul S. Antiplasmodial triterpenes from twigs of *Gardenia saxatilis*. Journal of Ethnopharmacology 2003; 88: 275-277.

Süzgec S, Meriçli AH, Houghton PJ, Çubukçu B. Flavonoids of *Helichrysum compactum* and their antioxidant and antibacterial activity. Fitoterapia 2005; 76: 269-272.

Swartz ME. 2005. Ultra-Performance Liquid Chromatography (UPLC): An Introduction. Separation Science Redefined. www.chromatographyonline.com, accessed on the 20th June 2008.

Swaziland Ministry of Health. 2000. 7th HIV Sentinel Serosurveillance Report: 2000 Ministry of Health and Social Welfare, Swaziland National AIDS/STDs Programme, Mbabane, October, unpublished report.

Tanaka M, Kuei CW, Nagashima Y, Taguchi T. Application of antioxidative maillrad reaction products from histidine and glucose to sardine products. Nippon Suisana Gakkaishil 1998; 54: 1409-1414.

Taylor JLS, Elgorashi EE, Maes A, van Gorp U, de Kimpe N, van Staden J, Verschaeve L. Investigating the safety of plants used in South African traditional medicine: Testing for genotoxicity in the micronucleus and alkaline comet assays. Environmental and Molecular Mutagenesis 2003; 42: 144-154.

Taylor S, Berridge V. Medicinal plants and malaria: an historical case study of research at the London School of Hygiene and Tropical Medicine in the twentieth century. Transactions of the Royal Society of Tropical Medicine and Hygiene 2006; 100: 707-714.

Tetali P, Waghchaure C, Daswani PG, Antia NH, Birdi TJ. Ethnobotanical survey of antidiarrhoeal plants of Parinche valley, Pune district, Maharashtra, India. Journal of Ethnopharmacology 2009; 123: 229-236.

Thomas EL, Lehrer RI, Rest RF. Human neutrophil antimicrobial activity. Reviews of Infectious Diseases. 1988; 10: S450-S456.

Tiwari OP, Tripathi YB. Antioxidant properties of different fractions of *Vitex negundo* Linn. Food Chemistry 2007; 100: 1170-1176.

Todar K. 2008. Todar's Online Textbook of Bacteriology.
www.textbookofbacteriology.net, accessed on the 30th March 2008.

Trager W and Jensen JB. Human malaria parasites in continuous culture. Science 1976; 190: 792-794.

Tsai TR, Tseng TY, Chenc CF, Tsai TH. Identification and determination of geniposide contained in *Gardenia jasminoides* and in two preparations of mixed traditional Chinese medicines. Journal of Chromatography A 2002; 961: 83-88.

Tshikalange TE, Meyer JJM, Hussein AA. Antimicrobial activity, toxicity and the isolation of a bioactive compound from plants used to treat sexually transmitted diseases. Journal of Ethnopharmacology 2005; 96: 515-519.

Turkoglu A, Kivrak I, Mercan N, Duru ME, Gezer K, Turkoglu H. Antioxidant and antimicrobial activities of *Morchella conica* Pers. African Journal of Biotechnology 2006; 5: 1146-1150.

Turrens JF. Oxidative stress and antioxidant defenses: a target for the treatment of diseases caused by parasitic protozoa. Molecular Aspects of Medicine 2004; 25: 211-220.

Tuyen NV, Kim DSHL, Fong HS, Soejarto DD, Khanh TC, Tri MV, Xuan LT. Structure elucidation of two triterpenoids from *Ficus fistulosa*. Phytochemistry 1998; 50: 467-469.

United Nations Development Programme (UNDP). Sharing innovative experiences: Examples of the developments of pharmaceutical products from medicinal plants, 2005: 5-6.

University of Swaziland. 1999. Proceedings of the Symposium on African Medicinal and Indigenous Food Plants and the Role of Traditional Medicine in Health Care.

Valli G, Giardina EV. Benefits, adverse effects and drug interactions of herbal therapies with cardiovascular effects. Journal of the American College of Cardiology 2002; 39: 1083-1095.

van Staden J. Ethnobotany in South Africa. Journal of Ethnopharmacology 2008; 119: 329-330.

van Vuueren SF. Antimicrobial activity of South African medicinal plants. Journal of Ethnopharmacology 2008; 119: 462-472.

van Vuuren SF, Viljoen AM, van Zyl RL, van Heerden FR, Başer KHC. The antimicrobial, antimalarial and toxicity profiles of helihumulone, leaf essential oil and extracts of *Helichrysum cymosum* (L.) D. Don subsp. *cymosum*. South African Journal of Botany 2006; 72: 287-290.

van Wyk BE, van Oudtshoorn B, Gericke N. 2000. Medicinal plants of South Africa. Briza Publications, Pretoria, South Africa

van Wyk BE, van Oudtshoorn B, Gericke N. 1997. Medicinal plants of Southern Africa. Briza Publications, Pretoria, South Africa.

van Zyl RL, Viljoen AM. *In vitro* activity of Aloe extracts against *Plasmodium falciparum*. South African Journal of Botany 2002; 68: 106-110.

van Zyl RL, Seatlholo ST, van Vuuren SF, Viljoen AM. The biological activities of 20 nature identical essential oil constituents. Journal of Essential Oil Research 2006; 18: 129-133.

van Zyl RL. Iron and malaria 2007 PhD. University of the Witwatersrand, Johannesburg, South Africa.

Vangapandu S, Jain M, Kaur K, Patil P, Patel SR, Jain R. Recent advances in antimalarial drug development. Medicinal Research Reviews 2007; 27: 65-107.

Veberic R, Colaric M, Stampar F. Phenolic acids and flavonoids of fig fruit (*Ficus carica* L.) in the northern Mediterranean region. Food Chemistry 2008; 106: 153-157.

Venter F and Venter JA. 2002. Making the most of indigenous trees, 2nd edition. Briza publications, Pretoria, South Africa.

Verpoote R, Dihal PP. Medicinal plants o of Surinam IV. Antimicrobial activity of some medicinal plants. Journal of Ethnopharmacology 1987; 21: 315-318.

Verschaeve L, Kestens V, Taylor JLS, Elgorashi EE, Maes A, Van Puyvelde L, De Kimpe N, Van Staden J. Investigation of the antimutagenic effects of selected South African medicinal plant extracts. Toxicology in Vitro 2004; 18: 29-35.

Veigas JM, Narayan MS, Laxman PM, Neelwarne B. Chemical nature, stability and bioefficacies of anthocyanins from fruit peel of *Syzygium cumini* Skeels. Food Chemistry 2007; 105: 619-627.

Viljoen AM, Subramoney S, van Vuuren SF, Baser KHC, Demirci B. The composition, geographical variation and antimicrobial activity of *Lippia javanica* (Verbenaceae) leaf essential oils. Journal of Ethnopharmacology 2005; 96: 271-277.

Vuong C, Otto M. *Staphylococcus epidermidis* infections. Microbes and Infections 2002; 4: 481-489.

Waako PJ, Smith P, Folb PI. *In vitro* interactions of *Aspilia africana* (Pers.) C.D. Adams, a traditional antimalarial medicinal plant, with artemisinin against *Plasmodium falciparum*. Journal of Ethnopharmacology 2005; 102: 262-268.

Wagner H, Bladt S. 1986. Plant Drug Analysis. A thin layer chromatography atlas. Springer.

Wang SC, Tseng TY, Hung CM, Tsai TH. Gardenia herbal active constituents: applicable separation procedures. Journal of Chromatography B 2004; 812: 193-202.

Warnock DW. 2007. Fungi superficial, subcutaneous and systemic infections. In: Greenwood D, Slack R, Peutherer J, Barer M. (Eds) Medical Microbiology, 7th edition. Churchill Livingstone, Elsevier.

Watt JM and Breyer-Brandwijk MG. 1962. The medicinal and poisonous plants of Southern and Eastern Africa, 2nd edition. Livingstone Ltd, London.

Wichi HP. Enhanced tumour development by butylated hydroxyanisole (BHA) from the prospective effect on forestomach and oesophageal squamous epithelium. Food Chemistry and Toxicology 1988; 26: 717-723.

Williamson EM. Synergy and other interactions in phytomedicine. Phytomedicine 2001; 5: 401-409.

Wojdylo A, Oszmianski J, Czemerys R. Antioxidant activity and phenolic compounds in 32 selected herbs. Food Chemistry 2007; 105: 940-949.

Wollenweber E, Stevens JF, Ivancic M**.** Flavonoid aglycones and a thiophene derivative from *Helichrysum cassianum*. Phytochemistry 1998; 47: 1441-1443.

Wong CC, Li HB, Cheng KW, Chen F. A systematic survey of antioxidant activity of 30 Chinese medicinal plants using the ferric reducing antioxidant power assay. Food Chemistry 2006; 97: 705-711.

Wong KC, Lai FY. Volatile constituents from the fruits of four *Syzygium* species grown in Malaysia. Flavour and Fragrance Journal 1996; 11: 61-66.

Word Health Organization (WHO). The promotion and development of traditional medicine technical report series no. 615, 1978; WHO Geneva.

World Health Organization (WHO). Fifty-sixth World Health Assembly 2003 A56/18.

World Health Organization (WHO). The World Health Report. Making a difference: Rolling back malaria, 1999; 49-61. WHO Geneva.

World Health Organization (WHO). World Malaria Report, 2008. WHO Geneva.

Wright CW. Traditional antimalarials and the development of novel antimalarial drugs. Journal of Ethonopharmacology 2005; 100: 67-71.

Yahnbaş B, Özdemir A, Selamet U, Eres M, Berna M, Yildiz B. Multi-organ toxicity following ingestion of mixed herbal preparations: An unusual but dangerous adverse effect of phytotherapy. European Journal of Medicine 2006; 17: 130-132.

Yamano T, Tsujimoto Y, Noda T, Shimizu M, Ohmori M, Morita S, Yamada. Hepatotoxicity of gardenia yellow colour in rats. Toxicology Letters 1988; 44: 177-182.

Yen GC, Duh PD, Tsai CL. Relationship between antioxidant activity and maturity of peanut hulls. Journal of Agricultural and Food Chemistry 2003; 41: 67-70.

Yff BTS, Lindsey KL, Taylor MB, Erasmus DG, Jäger AK. The pharmacological screening of *Pentanisia prunelloides* and the isolation of the antibacterial compound palmitic acid. Journal of Ethnopharmacology 2002; 79: 101-110.

Yoshioka N. Gonmori K, Tagshira A, Boonhooi O, Hayashi M, Saito Y, Mizugaki M. A case of aconitine poisoning with analysis of aconitine alkaloids by GC / SIM. Forensic Science International 1996; 81: 117-123.

Zhan Y, Dog CH, Yao YJ. Antioxidant activities of aqueous extract from cultivated fruit-bodies of *Cordyceps militaris* (L.) link *in vitro*. Journal of Integrative Plant Biology 2006: 48; 1365-1370.

Zhao WM, Xu JP, Qin GW, Xu RS. Two monoterpenes from fruits of *Gardenia jasminoides*. Phytochemistry 1994; 37: 1079-1081.

Zhu QY, Hackman RM, Ensunsa JL, Holt RR, Keen CL. Antioxidative activities of oolong tea. Journal of Agricultural and Food Chemistry 2002; 50: 6929-6934.

Ziaratnia SM, Hussein AA, Kiyoshi O, Muranaka T, Kunerta KJ, Meyer JJM. A novel phenolic compound and an alternative biosynthetic pathway for flavonoids in *Helichrysum aureonitens*. South African Journal of Botany 2008; 74: 384.

Zirihi GN, Mamby L, Guédé-Guina F, Bodo B, Grellier P. *In vitro* antiplasmodial activity and cytotoxicity of 33 West African plants used for the treatment of malaria. Journal of Ethnopharmacology 2005; 98: 281-285.

APPENDIX A: ABSTRACTS OF CONFERENCES AND PRESENTATIONS

Appendix A1: (Oral presentation: Indigenous Plant Use Forum, Johannesburg 2007; and Faculty Research Day, 2007)

Antimicrobial and toxicity studies of Swazi medicinal plants

SIBANDZE, G.F.[1], VAN ZYL, R.L.[1], VAN VUUREN, S.F.[1], VILJOEN, A.M.[2], AND AMUSAN, O.O.G.[3]

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Traditional medicine is widely used as a source of primary healthcare in most societies in Africa, including Swaziland. Traditional healers use various plants and combinations thereof to manage microbial infections that affect the skin, respiratory and gastro-intestinal systems. With the increasing prevalence of antibiotic-resistant microorganisms, novel drugs could be sourced from traditionally used phytomedicines. In consultation with Swazi traditional healers, fifteen Swazi plants were selected and the antimicrobial activity of 33 dichloromethane-methanol extracts were determined using the microplate dilution technique. Four bacterial strains, namely *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 2223), *Escherichia coli* (ATCC 25922) and *Klebsiella pneumoniae* (NCTC 9633) as well as the yeast, *Candida albicans* (ATCC 10231) were investigated. The leaves of *Dichrostachys cinerea* and the bark of *Ozoroa sphaerocarpa* were found to be active against all five microorganisms with MIC values ranging from <0.01 to 1 mg/ml. The most active extracts against *S. epidermidis* and *C. albicans* were the fruits of *O. sphaerocarpa* (MIC = <0.01 mg/ml) and the leaves of *Ricinus communis* (MIC = 0.3 mg/ml), respectively. Whilst the least active plants were the roots of *Dicoma anomala* and fruit of *Gardenia spatulifolia* (MIC range = 4 to >16 mg/ml). The synergistic interaction observed between *Syzygium cordatum* and *O. sphaerocarpa* against *E. coli* supports the rationale by traditional healers to use these plants in combination. The cytotoxicity of these plants as determined by the MTT tetrazolium-based assay on human kidney epithelial cells will be presented in correlation with their respective antimicrobial activity.

Appendix A2: (Oral and Poster Presentation: South African Pharmacology and Toxicology Congress, Buffelspoort, 2007; and Poster Presentation at The School of Therapeutic Science Research Day, 2008)

Pharmacological Properties of Swazi Medicinal Plants

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Purpose:

Traditional medicine is widely used as a source of primary healthcare for more than 80% of the population of Swaziland. Traditional healers use various plants and combinations thereof to manage different ailments. With the increasing prevalence of antibiotic-resistant microorganisms, novel drugs could be sourced from traditionally used phytomedicines. In consultation with Swazi traditional healers, fifteen Swazi plants were selected and screened for their, antimicrobial and anti-oxidant activities and toxicity profiles.

Methods:

The plants were collected from the Manzini region in Swaziland, air-dried at room temperature and extracted with dichloromethane/methanol (1:1). Ultra Performance Liquid Chromatography was done to determine chemical profiles of the extracts. Antimicrobial activity was determined using the minimum inhibitory concentrations assay against *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 2223), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (NCTC 9633) and *Candida albicans* (ATCC 10231). The DPPH (2,2-diphenyl--picryl-hydrazil) scavenging activity and ferrous metal chelating activity were correlated to the total phenolic content of the plant extracts. Toxicity profiles of the extracts were tested on human kidney epithelial cells using the tetrazolium-based MTT viability assay.

Results:

A number of the plant extracts (51.52%) displayed good antimicrobial activity with MIC values ranging from 0.0013 to 1.00 mg/ml. The fruit of *Ozoroa sphaerocarpa* and the leaves of *Dichrostachys cinerea* were the most active especially against *S. epidermidis*. The synergistic interaction observed between *Syzygium cordatum* and *O. sphaerocarpa* against *E. coli* supports the rationale by traditional healers to use these plants in combination. *D. cinerea* (leaves) was the most active free radical scavenger (IC_{50}: 5.89 ± 0.39 µg/ml) as compared to ascorbic acid IC_{50}: 5.61 ± 1.13 µg/ml). For approximately 70% of the plants, there was a correlation between DPPH scavenging and ferrous metal chelating activity. The toxicity profile of the plant extracts indicated that about 40% of the extracts had IC_{50} values greater than 100 µg/ml.

Appendix A3: (Poster Presentation: World Conference on Medicinal and Aromatic Plants IV, Cape Town 2008)

The antimalarial and toxicity studies of Swazi medicinal plants

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Malaria, caused by *Plasmodium falciparum*, is a major cause of morbidity and mortality in sub-Saharan Africa. Many of the standard antimalarial drugs have become ineffective due to the parasite developing resistance against them. Traditional medicine is a source of primary healthcare in most societies in Africa, with about 80 % of the people consulting traditional medical practitioners for healthcare. Traditional healers use various medicinal plants to treat malaria according to its "flu-like" symptoms and fever. In consultation with Swazi traditional healers, fifteen Swazi plants were selected and screened for their antimalarial activity and toxicity profiles. The plants were collected from the Manzini region in Swaziland, air-dried and extracted with dichloromethane: methanol (1:1). Antimalarial activity against *P. falciparum* was determined using the tritiated hypoxanthine incorporation assay. Toxicity profiles of the extracts were tested on human red blood cells and kidney epithelial cells. Of the 33 extracts tested, 5 displayed *in vitro* antimalarial activity with IC_{50} values less than 20 µg/ml, namely *Terminalia phanerophlebia* (leaves), *Berkheya setifera* (stem/root), *Priva meyeri* (whole plant), *Trichilia emetica* (leaves) and *Breonadia salicina* (bark). The five most active extracts did not cause red blood cell haemolysis at concentrations ten times greater than the IC_{50}. Similarly, *T. phanerophlebia*, *B. setifera* and *B. salicina* were not toxic to the kidney epithelial cells. However, *P. meyeri* and *T. emetica* displayed toxicity (IC_{50} = 14.633 ± 1.616; 45.945 ± 5.104 µg/ml), respectively. The study supports the traditional use of some of the plants in the treatment of the "flu-like" and fever symptoms of malaria.

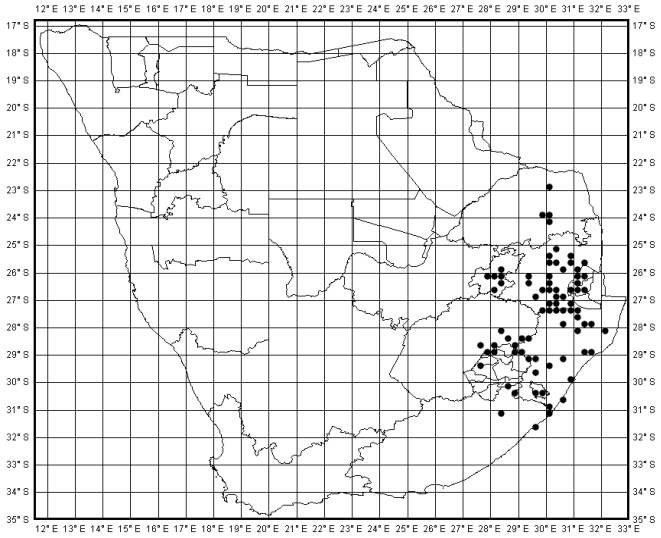
APPENDIX B

Plant Monographs

B1. *Berkheya setifera* DC.

Scientific name: *Berkheya setifera* DC.
Family: Astereaceae
Vernacular names*: lulwimi lwenkhomo* (Swati)

Berkheya setifera flower



Distribution map for *Berkheya setifera*

Botanical description
This is a perennial herb with leaves that are alternate and spiny. The capitulum is heterogamous and radiate or homogamous and discoid. The phyllaries are in several series, connate at the base only, always spiny, but spines may be slender and bristle-like. The ray florets are yellow (Watt and Breyer-Brandwijk, 1962).

Distribution
The plant is distributed from Tanzania to KwaZulu-Natal, South Africa.

Medicinal use
Together with *Dicoma anomala*, the root infusion is used for toothache, sterility, jaundice, biliousness, urinary problems, burns, boils, skin conditions and abdominal disorders (Watt and Breyer-Brandwijk, 1962; Amusan *et al*., 2002; Adeniji *et al*., 2000). *Berkheya speciosa* is used for the treatment of schistosomiasis (Sparg *et al*., 2000).

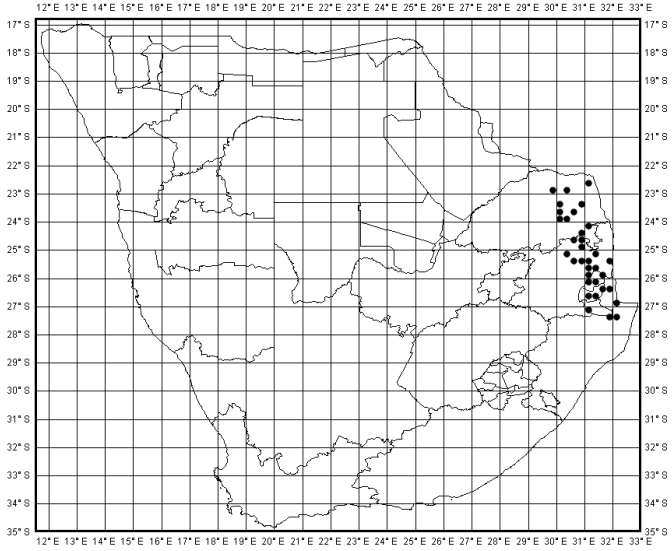
Pharmacological properties of *B. setifera*.

Pharmacological properties	Flower	Leaves	Stem/root
3H- hypoxanthine incorporation assay (IC_{50} in µg/ml ± SD)	32.3 ± 0.74	66.40 ± 4.19	15.12 ± 1.80
Red blood cell lysis	>100 (0.47 ± -0.021%)	>100 (2.53 ± 0.07%)	178.41 ± 33.16
Human kidney epithelial cell toxicity	>200 (53.07 ± 3.80%)	>200 (68.71 ± 5.97%)	101.79 ± 8.42
Staphylococcus aureus (MIC in mg/ml)	4.00	3.33	4.00
Staphylococcus epidermidis (MIC in mg/ml)	4.00	1.67	8.00
Escherichia coli (MIC in mg/ml)	3.00	5.33	2.00
Klebsiella pneumoniae (MIC in mg/ml)	3.33	1.33	1.33
Candida albicans (MIC in mg/ml)	2.00	0.67	1.33
DPPH free radical scavenging (IC_{50} in µg/ml ± SD)	>100	>100	>100
Metal chelating activity (IC_{50} in µg/ml± SD)	>100	>100	>100
Lipid peroxidation (percent/100 µg/ml)	24.66 ± 4.38	2.54 ± 0.47	59.45 ± 2.09
Total phenolic content (mg GAE/g extract)	243.00 ± 3.03	260.73 ± 6.41	443.93 ± 44.71
Flavonoid content (mg RE/g extract)	13.00 ± 0.60	<0.010	<0.010

B2. *Breonadia salicina* (Vahl) Hepper & J.R.I. Wood

Scientific name: *Breonadia salicina* (Vahl) Hepper & J.R.I. Wood
Family: Rubiaceae
Vernacular names: redwood (English), *umhlume* (Swati)

Breonadia salicina



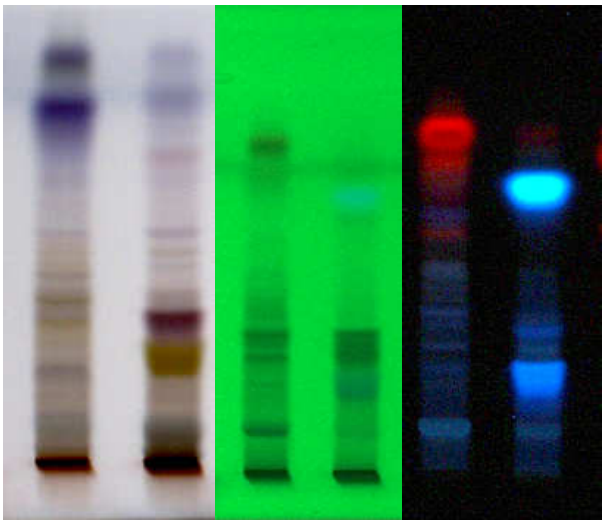
Breonadia salicina distribution map

Botanical description
This is a savannah streamside tree growing up to 20 m in height with a dense irregular crown. The leaves are narrow and arranged in whorls at the end of the branchlets. From December to March, the flower heads appear as long-stalked creamy, yellowish, pinkish, pale mauve, sweet smelling globular heads and the fruit is composed of numerous small 2-celled fruitlets in a head of 6 mm in diameter. It is mostly found on stream banks in savannahs. The wood is used for boat-building, furniture and beams, while the bark and roots are ingredients of a hunting poison in north Nigeria.

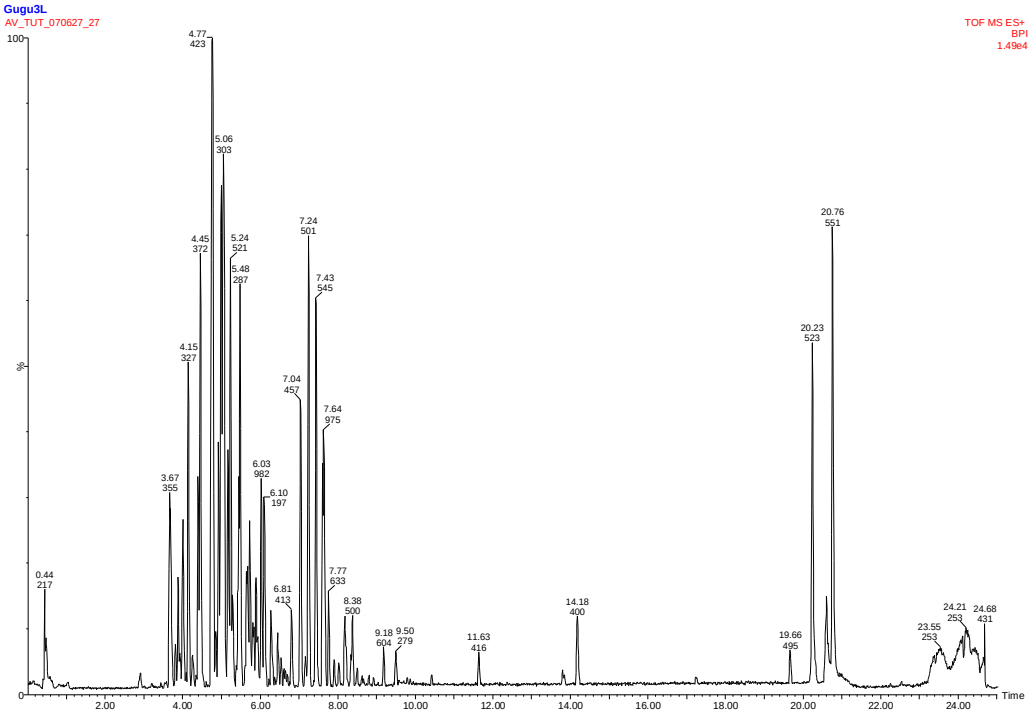
Distribution
The plant is widely distributed from Tanzania in the north to KwaZulu-Natal, South Africa in the south.

Medicinal use
Medicinally, a decoction of the bark is used to treat diarrhoea and colic, and the bark powder is sprinkled on injuries or wounds (Neuwinger, 1996; Venter and Venter, 2002).

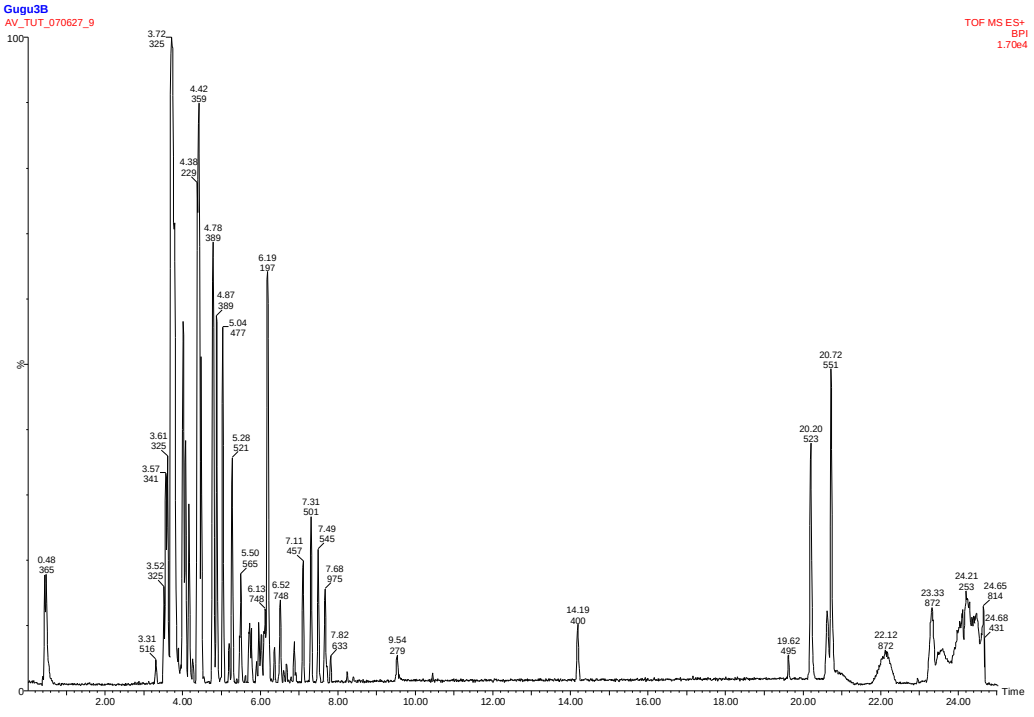
Phytochemistry
Alkaloids have been isolated in the twigs and leaves; triterpenes and saponins are found in stems and leaves; while the wood contains polyphenols and quinones. The stem bark is rich in tannins (Neuwinger, 1996).



B. salicina HPTLC chromatograms visualised after derivatization with vanillin-sulphuric acid and under 254 nm and 366 nm light, from left; leaves and bark.



(A)



(B)

Base peak intensity chromatograms for *B. salicina*; leaves (A) and bark (B).

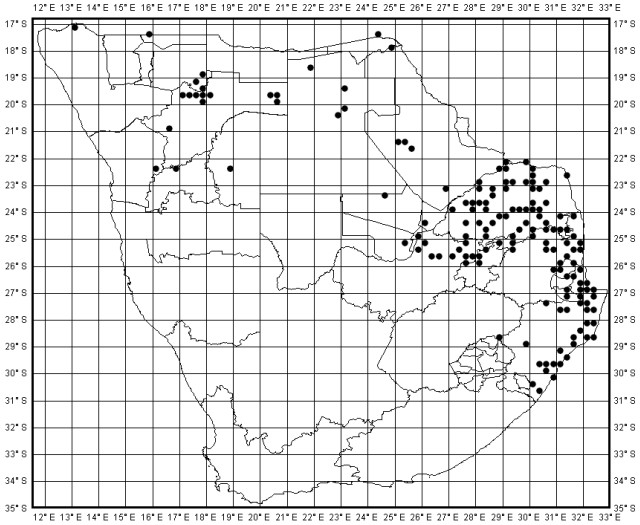
Pharmacological properties of *B. salicina*.

Pharmacological properties	Leaves	Bark
3H- hypoxanthine incorporation assay (IC_{50} in µg/ml ± SD)	135.79 ± 10.75	7.41 ± 1.88
Red blood cell lysis	>200 (20.31 ± 2.61%)	>100 (9.93 ± 3.73%)
Human kidney epithelial cell toxicity	182.66 ± 12.44	>200 (70.84 ± 2.72%)
Staphylococcus aureus (MIC in mg/ml)	5.33	8.00
Staphylococcus epidermidis (MIC in mg/ml)	1.33	4.00
Escherichia coli (MIC in mg/ml)	1.33	11.00
Klebsiella pneumoniae (MIC in mg/ml)	2.67	2.67
Candida albicans (MIC in mg/ml)	2.67	5.33
DPPH free radical scavenging (IC_{50} in µg/ml ± SD)	25.86 ± 2.86	24.91 ± 3.14
Metal chelating activity (IC_{50} in µg/ml± SD)	>100	>100
Lipid peroxidation (percent/100 µg/ml)	58.97 ± 2.65	70.72 ± 1.39
Total phenolic content (mg GAE/g extract)	142.40 ± 4.00	<0.010
Flavonoid content (mg RE/g extract)	12.20 ± 1.97	3.40 ± 0.20

B3. *Dichrostachys cinerea* (L.) Wight & Arn

Scientific name: *Dichrostachys cinerea* (L.) Wight & Arn
Family: Mimosaceae
Vernacular names: sickle bush (English), *lusekwane* (Swati)

Dichrostachys cinerea flower



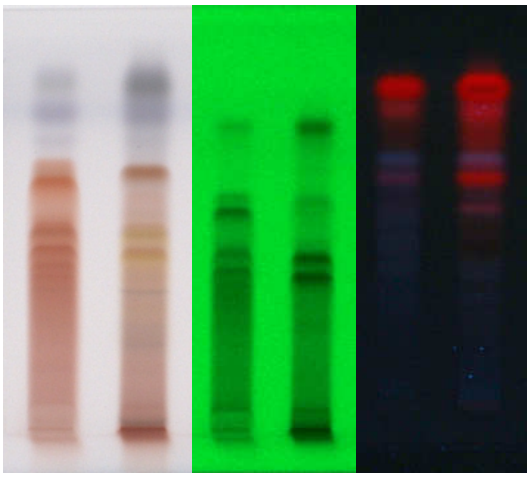
Dichrostachys cinerea distribution map

Botanical description
This is a semi-deciduous to deciduous tree growing up to 7 m tall with an open crown. The leaves are twice-compound ending in two leaflets with 20-27 pairs of leaflets each. The flowers appear from October to February and are a pendulous 40-50 mm long, two-coloured spikes, with the upper part being pink and lower part yellow (Watt and Breyer-Brandwijk, 1962; Venter and Venter, 2002).

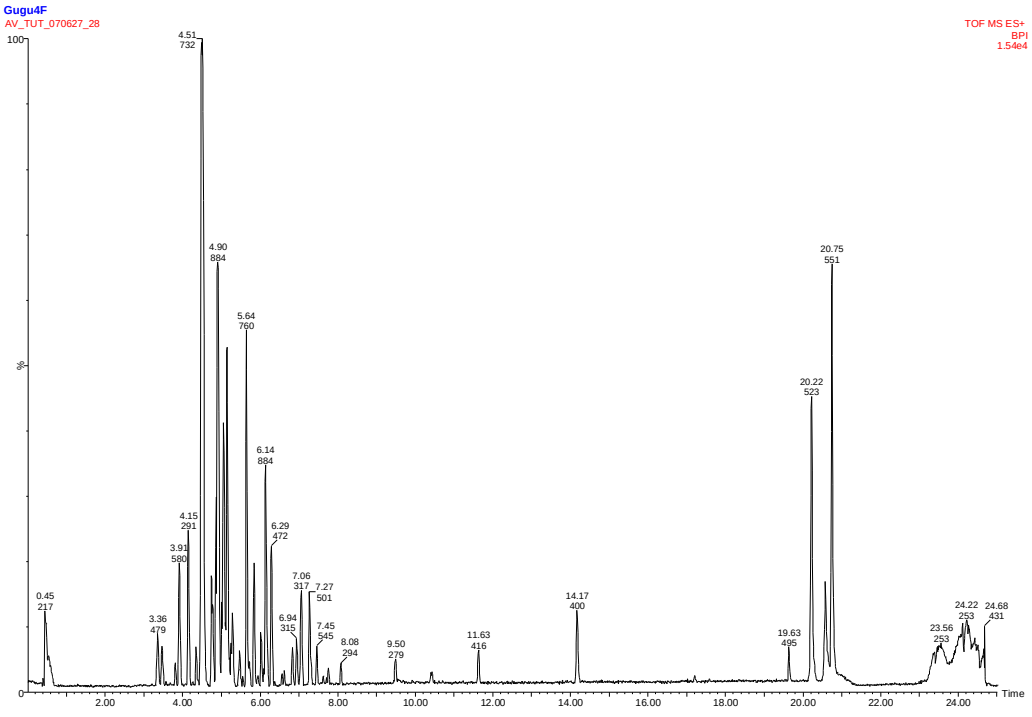
Distribution
The shrub has a wide distribution from Ethopia to KwaZulu-Natal, South Africa in the south. It predominantly grows in woodland, forest margins and grassland.

Medicinal use
The bark is used to treat elephantiasis which is caused by parasitic worms, such as *Wuchereria bancrofti*, *Brugia malayi*, and *B. timori*; while the root is used for toothache, stomach troubles, indigestion/diarrhoea, rheumatism and chest complaints. The burnt twigs are used for bone fractures and sexually transmitted diseases and the leaf infusion for skin infections, postpartum pain, bronchitis, abdominal pains, epilepsy and snake bite (Kambizi and Afolayan, 2001; Eisa *et al*., 2000; Watt and Breyer-Brandwijk, 1962; Venter and Venter, 2002).

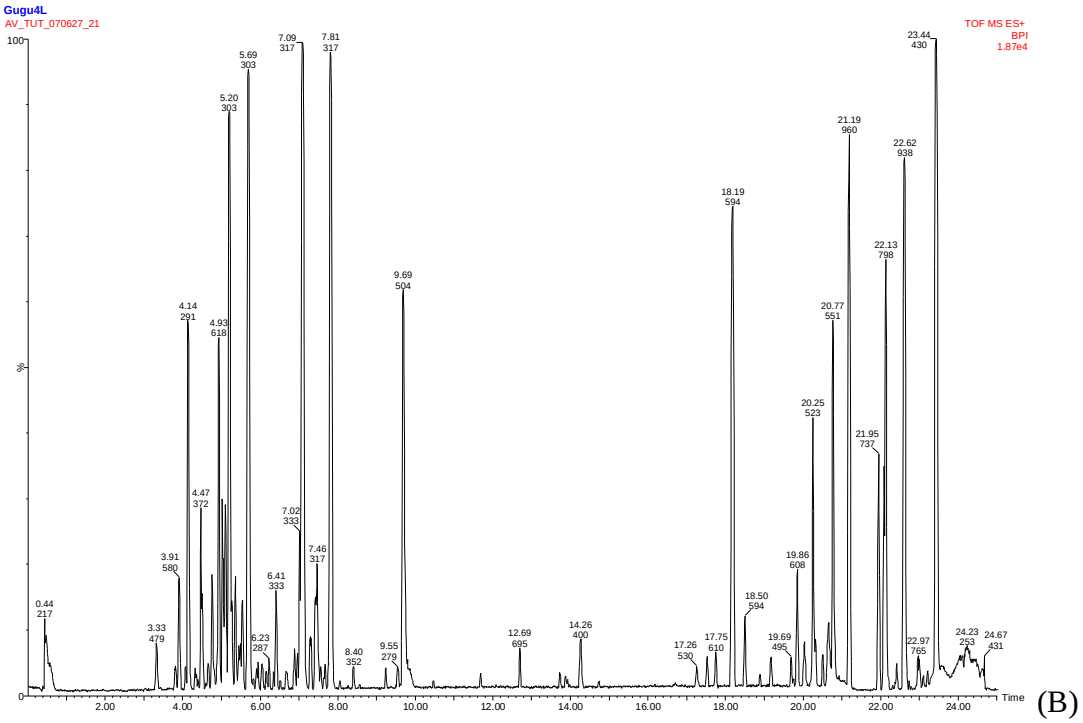
Phtochemistry
The aeriel parts of *D. cinerea* contain hentriacontanol, β-amyrin and sitosterol. The bark contains friedelin, α-amyrin and sitosterol; whilst the heartwood contains octacosanol (Joshi and Sharma, 1974). Other compounds that have been isolated from *D. cinerea* are (-)-mesquitol (2,3-*trans*-3',4'7,8-tetrahydroxyflavan-3-ol) and (-)-epicatechin (Rao *et al*., 2003).



D. cinerea HPTLC chromatograms visualised after derivatization with vanillin-sulphuric acid and under 254 nm and 366 nm light, from left; fruit and leaves.



(A)



(B)

Base peak intensity chromatograms for *D. cinerea*; fruit (A) and leaves (B).

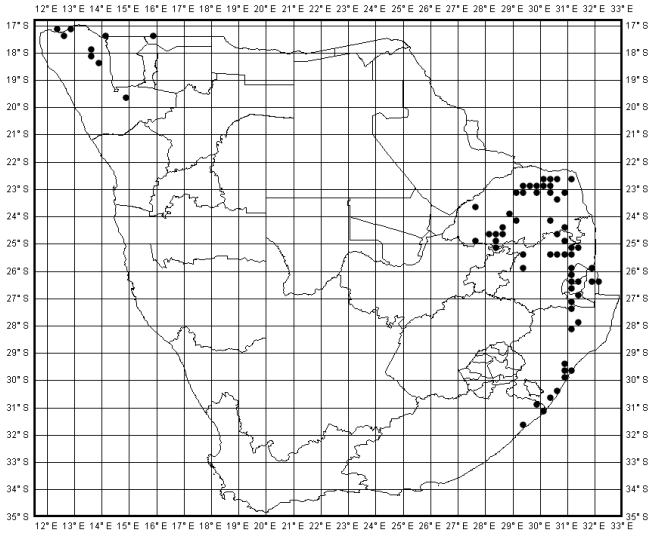
Pharmacological properties of *D. cinerea.*

Pharmacological properties	Fruit	Leaves
3H- hypoxanthine incorporation assay (IC_{50} in µg/ml ± SD)	26.51 ± 1.37	106.76 ± 9.64
Red blood cell lysis	150.56 ± 20.48	>100 (13.92 ± 1.01%)
Human kidney epithelial cell toxicity	65.33 ± 5.00	139.19 ± 7.07
Staphylococcus aureus (MIC in mg/ml)	0.29	0.44
Staphylococcus epidermidis (MIC in mg/ml)	0.38	0.42
Escherichia coli (MIC in mg/ml)	0.25	0.50
Klebsiella pneumoniae (MIC in mg/ml)	0.56	0.67
Candida albicans (MIC in mg/ml)	0.67	0.42
DPPH free radical scavenging (IC_{50} in µg/ml ± SD)	6.02 ± 0.79	5.89 ± 0.39
Metal chelating activity (IC_{50} in µg/ml± SD)	17.37 ± 0.77	36.27 ± 3.01
Lipid peroxidation (percent/100 µg/ml)	95.03 ± 3.25	60.68 ± 2.94
Total phenolic content (mg GAE/g extract)	51.90 ± 3.07	<0.010
Flavonoid content (mg RE/g extract)	2.10 ± 0.27	6.27 ± 0.61

B4. *Ficus glumosa* Delile

Scientific name: *Ficus glumosa* Delile
Synonyms: *Ficus sonderi*
Family: Moraceae
Vernacular names: mountain fig (English), *umkhiwa* (Swati)

Ficus glumosa



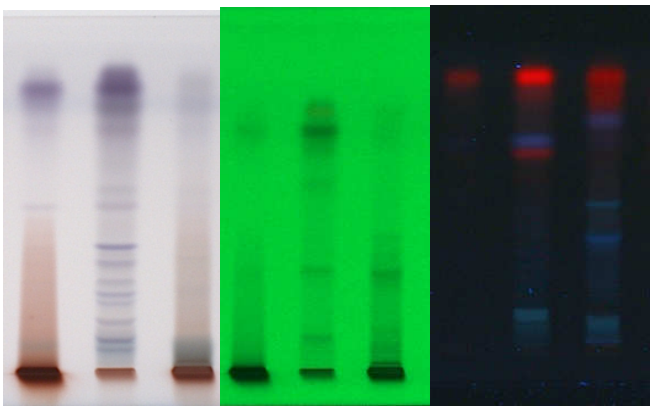
Ficus glumosa distribution map

Botanical description
It is moderate sized erect tree growing up to 15 m in height, densely leafed and usually evergreen. All parts of the tree produce non-toxic milky latex. Figs are numerous among the leaf axils and stem and spherical. Fruiting period is indefinite (Venter and Venter, 2002; Adeniji *et al*., 2000).

Distribution
The plant is distributed from Tanzania to KwaZulu-Natal, South Africa and found along drainage lines, rocky outcrops and cliffs in woodland and wooded grassland.

Medicinal use
The bark concoction is drunk for the treatment of diarrhoea and generalised malaise; while the milky latex is used to treat toothache, cuts, infections and sore eyes (de Boer *et al*., 2005; Venter and Venter, 2002; Adeniji *et al*., 2000).

Phytochemistry



F. glumosa HPTLC chromatograms visualised after derivatization with vanillin-sulphuric acid and under 254 nm and 366 nm light, from left; bark, fruit and leaves.

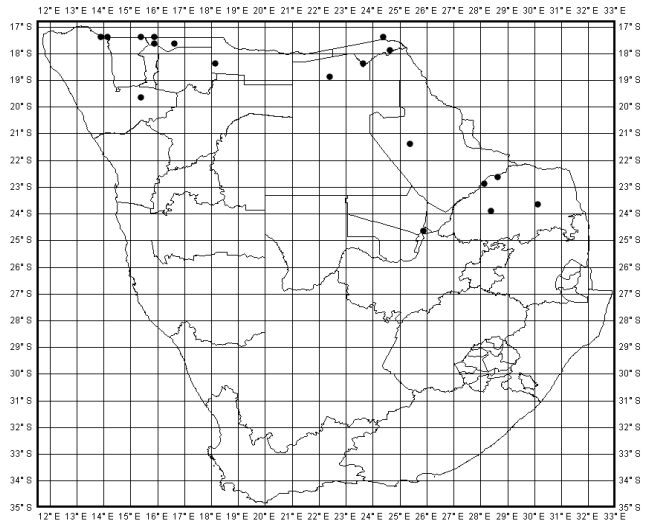
Pharmacological properties of *F. glumosa*.

Pharmacological properties	Fruit	Leaves	Bark
3H- hypoxanthine incorporation assay (IC_{50} in µg/ml ± SD)	117.54 ± 14.03	19.40 ± 2.20	>200 (74.84 ± 3.95%)
Red blood cell lysis	>100 (5.91 ± 1.03%)	>100 (27.43 ± 3.02%)	>100 (19.95 ± 1.33%)
Human kidney epithelial cell toxicity	189.11 ± 8.62	117.82 ± 2.56	40.56 ± 2.03
Staphylococcus aureus (MIC in mg/ml)	3.00	4.00	0.25
Staphylococcus epidermidis (MIC in mg/ml)	1.50	2.00	0.25
Escherichia coli (MIC in mg/ml)	2.00	2.00	0.25
Klebsiella pneumoniae (MIC in mg/ml)	2.00	1.33	5.33
Candida albicans (MIC in mg/ml)	2.00	3.33	5.33
DPPH free radical scavenging (IC_{50} in µg/ml ± SD)	70.09 ± 8.20	46.76 ± 0.64	5.84 ± 1.53
Metal chelating activity (IC_{50} in µg/ml± SD)	>100	>100	39.02 ± 3.41
Lipid peroxidation (percent/100 µg/ml)	55.44 ± 2.85	65.62 ± 3.78	12.61 ± 1.88
Total phenolic content (mg GAE/g extract)	45.90 ± 0.78	<0.010	<0.010
Flavonoid content (mg RE/g extract)	14.07 ± 1.62	5.47 ± 0.12	0.78 ± 0.03

B5. *Gardenia volkensii* K. Schum subsp. *Spatulifolia*

Scientific name: *Gardenia volkensii* K. Schum subsp. *spatulifolia*
Family: Rubiaceae
Vernacular names: bushveld gardenia (English), *manyongwana* (Swati)

Gardenia spatulifolia



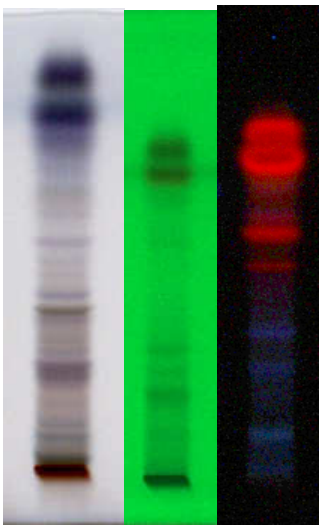
Gardenia spatulifolia distribution map

Botanical description
It is a semi-deciduous to evergreen tree, depending on habitat. It grows up to 8 m tall and has a roundish much branched crown. The leaves are in whorls of 3, crowded at tip of short stout branchlets and mostly spathe-shaped, glossy with an entire margin, midrib and secondary veins conspicuous on upper surface. The flower, appearing in July to December, is a single calyx ribbed with a short slit down one side and the corolla white and fragrant, turning yellow with age. The fruit is ovoid to spherical, shallowly ribbed, greyish-green and covered with grey warts (Venter and Venter, 2002).

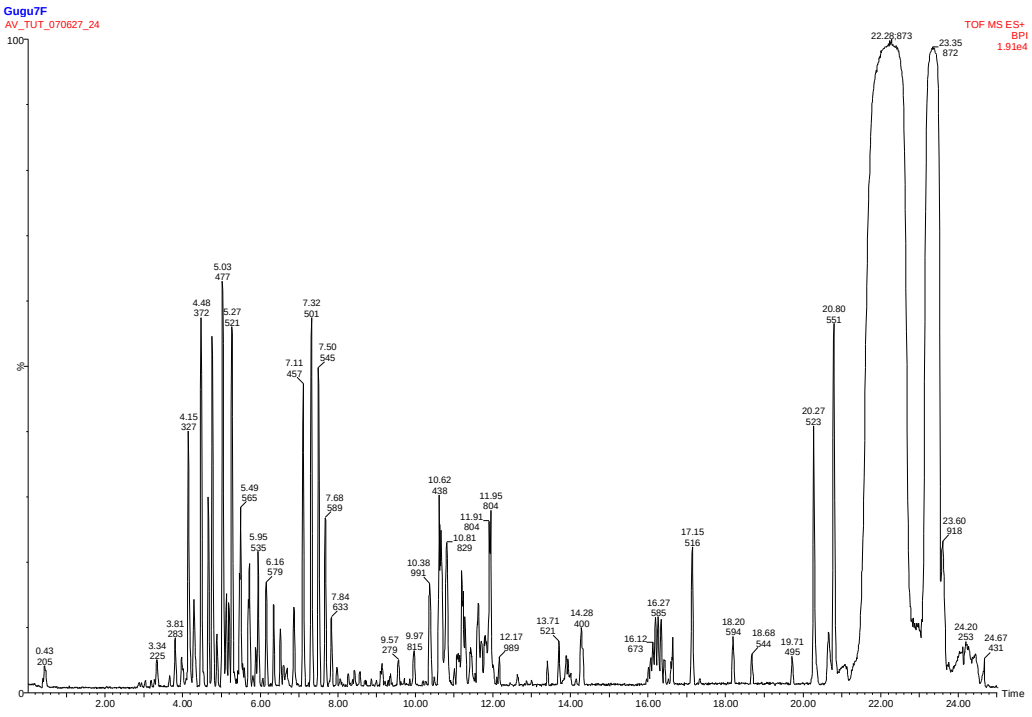
Distribution
The plant has a distribution from Angola in the north to KwaZulu-Natal, South Africa in the south and grows in woodlands.

Medicinal use
The fruit infusion is drunk to induce vomiting in the treatment of diabetes mellitus and other stomach troubles, while the burnt roots are used for pneumonia. A decoction of the root is used for epilepsy, headaches and earache (Venter and Venter, 2002; Verschaerve *et al*., 2004; Amusan *et al*., 2002; PP Ndlovu, personal communication). In Thailand and Ghana, *Gardenia* species are used for the treatment of malaria (Suksamrarn *et al*., 2003; Asase *et al*., 2005)

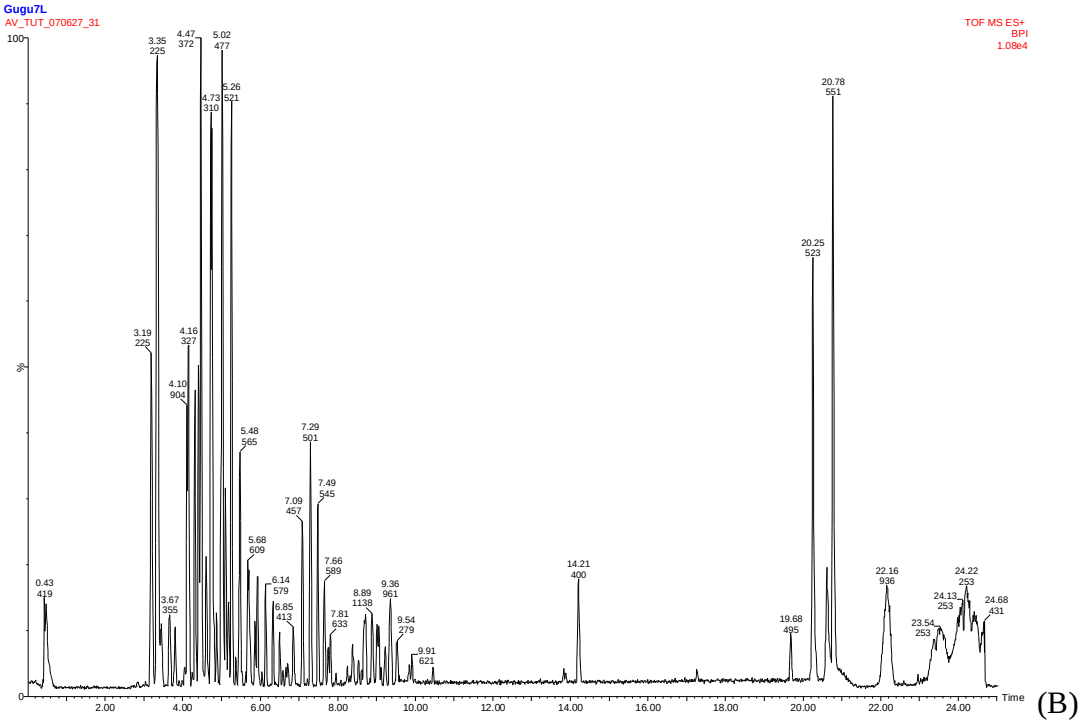
Phytochemistry



G. spatulifolia (leaves) HPTLC chromatograms visualised after derivatization with vanillin-sulphuric acid and under 254 nm and 366 nm light.



(A)



(B)

Base peak intensity chromatograms for *G. spatulifolia*; fruit (A) and leaves (B).

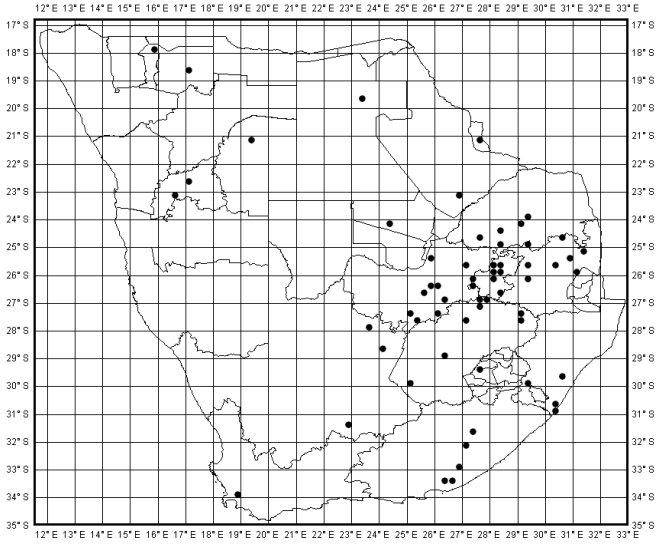
Pharmacological properties of *G. spatulifolia*.

Pharmacological properties	Fruit	Leaves
3H- hypoxanthine incorporation assay (IC_{50} in µg/ml ± SD)	124.90 ± 9.79	>200 (54.89 ± 4.84)
Red blood cell lysis	117.42 ± 10.96	>200 (16.01 ± 0.57%)
Human kidney epithelial cell toxicity	21.30 ± 2.67	89.46 ± 1.24
Staphylococcus aureus (MIC in mg/ml)	12.00	4.00
Staphylococcus epidermidis (MIC in mg/ml)	6.00	1.67
Escherichia coli (MIC in mg/ml)	8.00	2.00
Klebsiella pneumoniae (MIC in mg/ml)	8.00	1.33
Candida albicans (MIC in mg/ml)	12.00	0.39
DPPH free radical scavenging (IC_{50} in µg/ml ± SD)	>100	21.57 ± 4.00
Metal chelating activity (IC_{50} in µg/ml± SD)	>100	>100
Lipid peroxidation (percent/100 µg/ml)	58.24 ± 1.47	77.55 ± 2.58
Total phenolic content (mg GAE/g extract)	110.60± 1.06	228.30 ± 2.43
Flavonoid content (mg RE/g extract)	12.80 ± 1.40	2.53 ± 0.12

B6. *Guilleminea densa* var. *densa* (Willd.)moq.

Scientific name: *Guilleminea densa* var. *densa* (Willd.) moq.
Synonyms: *Brayulinea densa* Humb. & Bonpl. ex Willd
Family: Amaranthaceae
Vernacular names: *sanama* (Swati)

Guilleminea densa var. *densa*



Guilleminea densa distribution map

Botanical description
It is a prostrate or sometimes decumbent, mat-forming perennial herb with a rootstock considerably thickened for up to about 5 cm below the ground and then abruptly more slender, mat from about 7–70 cm across. The stems are numerous from the base, much-branched, branches are opposite (or alternate by reduction of one of the pair), more or less densely white-lanate. Leaves are variable in size and shape. The inflorescence is dense, ovoid, of up to about 10 flowers and whitish in colour (Aluka, 2008).

Distribution
Has a wide distribution throughout southern Africa.

Medicinal use
The root (tuber) is mixed with water and used to induce vomiting for the treatment of nausea (PP Ndlovu; personal communication).

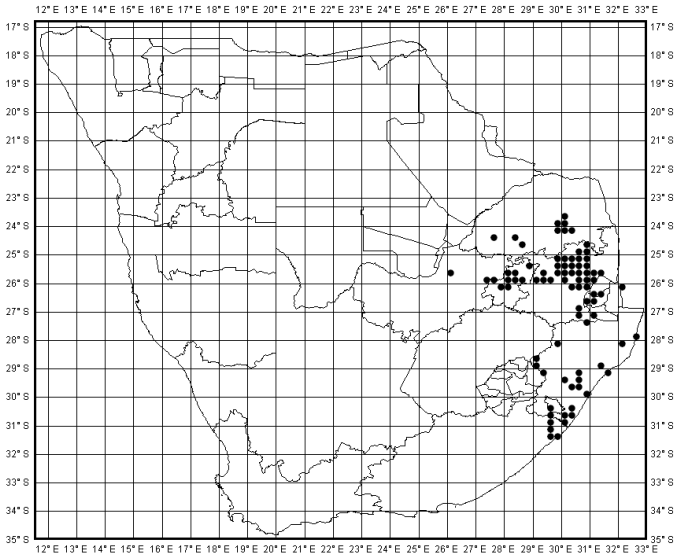
Pharmacological properties of *G. densa*.

Pharmacological properties	Whole plant
3H- incorporation assay (IC_{50} in µg/ml ± SD)	162.32 ±17.38
Red blood cell lysis	>200 (5.04 ± 0.28%)
Human kidney epithelial cell toxicity	>200 (77.58 ± 0.65 %)
Staphylococcus aureus (MIC in mg/ml)	4.00
Staphylococcus epidermidis (MIC in mg/ml)	1.25
Escherichia coli (MIC in mg/ml)	1.33
Klebsiella pneumoniae (MIC in mg/ml)	1.33
Candida albicans (MIC in mg/ml)	1.00
DPPH free radical scavenging (IC_{50} in µg/ml ± SD)	>100
Metal chelating activity (IC_{50} in µg/ml± SD)	>100
Lipid peroxidation (percent/100 µg/ml)	23.70 ± 3.80
Total phenolic content (mg GAE/g extract)	150.50 ± 0.99
Flavonoid content (mg RE/g extract)	<0.010

B7. *Helichrysum acutatum* DC.

Scientific name: *Helichrysum acutatum* DC.
Family: Astereaceae
Vernacular names: *Imboziso* (Swati)

Helichrysum acutatum

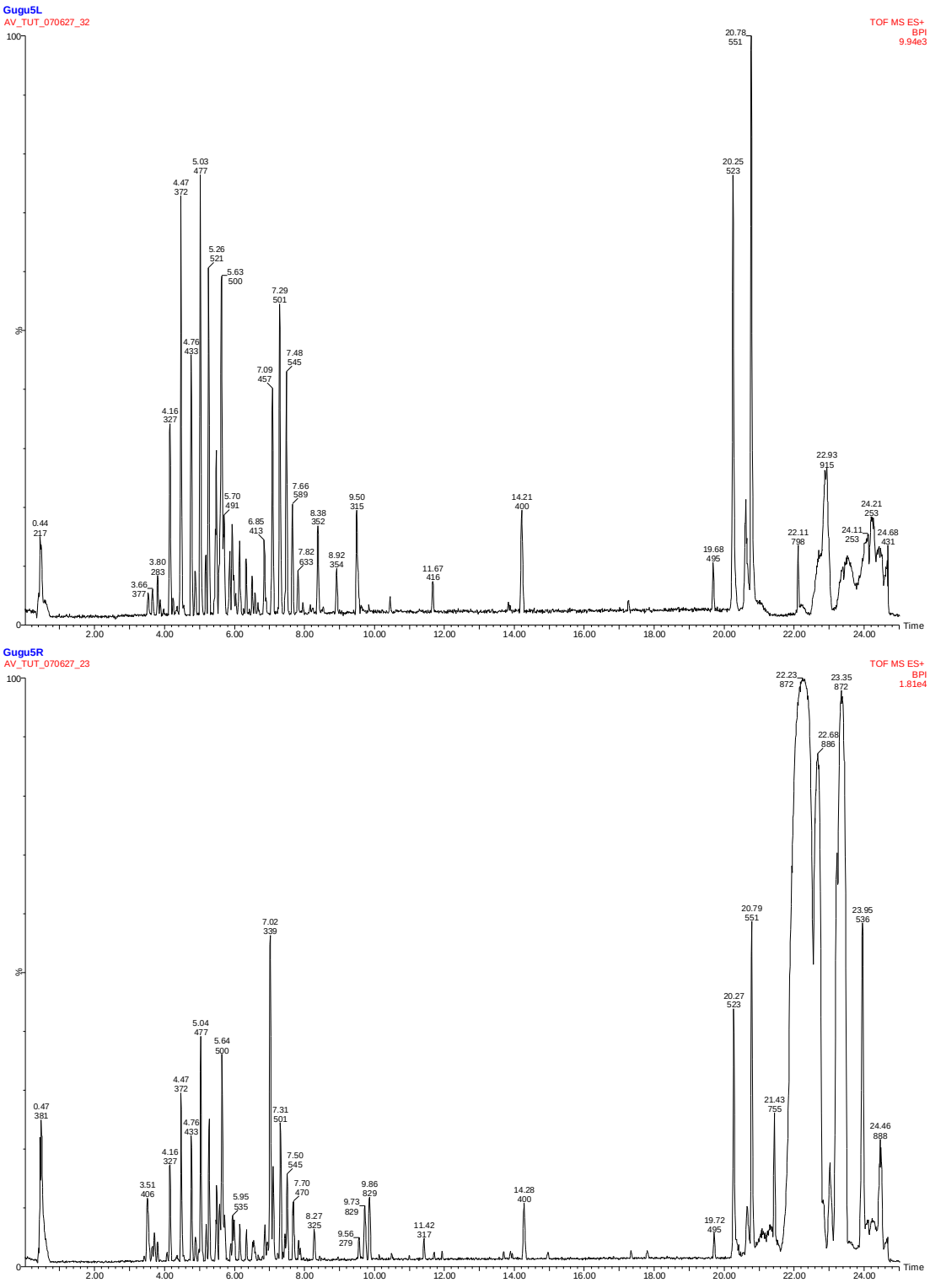


Helichrysum acutatum distribution map

Botanical description
This is a herbaceous plant with stout rootstalk and unbranched erect shoots up to about 60 cm in height. It is densely grey-woolly throughout. The leaves are few; stalk less, tapering to a broad base and to long fin-pointed tip. The inflorescence is on long common stalk, much-branched, compact, about 6 cm in diameter. The flower heads are bright yellow. The root is tuberous and fibrous (Compton, 1976; Adeniji *et al*., 2000).

Distribution
The plant is distributed from Tanzania to KwaZulu-Natal, South Africa.

Medicinal use
A concoction of the roots is used as an antidote for poison (PP Ndlovu; personal communication). Other uses of this genus include the treatment of topical infections, respiratory ailments and as a dressing in circumcision rites (van Vuuren *et al*., 2006). *Helichrysym* species (leaves and whole plant) are used for cough and tuberculosis (Watt and Breyer-Brandwijk, 1962). This genus has been reported to posses anticancer and antimycobacterial activity (Fouche *et al*., 2008; McGaw *et al*., 2008).



(A)

(B)

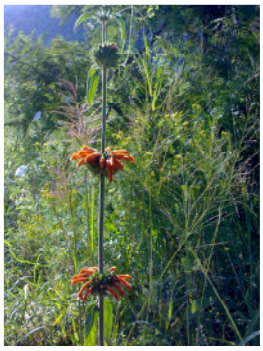
UPLC base peak intensity for *H. acutatum* leaves (A) and roots (B).

Pharmacological properties of *H. acutatum*.

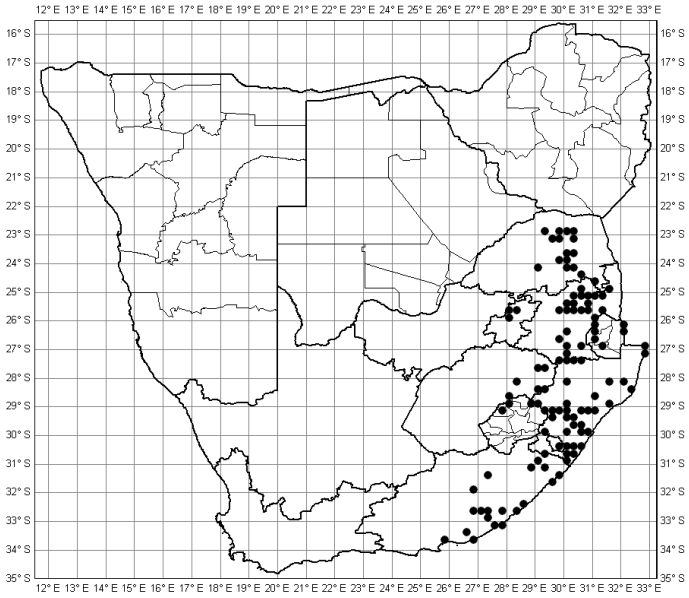
Pharmacological properties	Leaves	Roots
3H- incorporation assay (IC_{50} in µg/ml ± SD)	>200 (66.14 ± 7.69%)	>200 (76.67 ± 3.56%)
Red blood cell lysis	>200 (8.15 ± 3.67%)	>200 (1.34 ± 0.08%)
Human kidney epithelial cell toxicity	113.53 ± 6.91	>200 (78.71 ± 1.34%)
Staphylococcus aureus (MIC in mg/ml)	4.00	>16
Staphylococcus epidermidis (MIC in mg/ml)	1.67	13.33
Escherichia coli (MIC in mg/ml)	2.67	2.67
Klebsiella pneumoniae (MIC in mg/ml)	1.33	2.00
Candida albicans (MIC in mg/ml)	0.50	4.00
DPPH free radical scavenging (IC_{50} in µg/ml ± SD)	109.75 ± 9.50	>100
Metal chelating activity (IC_{50} in µg/ml± SD)	31.68 ± 2.23	>100
Lipid peroxidation (percent/100 µg/ml)	46.34 ± 3.04	51.67 ± 3.13
Total phenolic content (mg GAE/g extract)	182.80 ± 4.65	<0.010
Flavonoid content (mg RE/g extract)	0.50 ± 0.10	3.50 ± 0.10

B8. *Leonotis intermedia* Lindl.

Scientific name: *Leonotis intermedia* Lindl.
Family: Lamiaceae
Vernacular names: klipdagga (English), *tjwalabenyoni* (Swati)



Leonotis intermedia



Leonotis intermedia distribution map

Botanical description
This is a shrub that grows from 2-5 m in height with a thick woody base. All parts of the plant have a strong smell and the leaves are opposite each other on the stem, long and narrow, toothed in the upper part and distinctly hairy. The flowers are bright orange and tubular and borne in characteristic rounded groups (van Wyk *et al*., 2000).

Distribution
It has a wide distribution in the eastern parts of South Africa.

Medicinal use
The fruit and roots are ground into a powder and mixed with soft porridge in the treatment of cancer. A decoction of the roots or leaves of *Leonotis* spp in general is mainly used for toothache, snake bite, skin disease (twigs), muscular cramps, haemorrhoids, influenza, coughs, colds, indigestion, high blood pressure, bronchitis and also smoked as a narcotic in place of *Cannabis sativa* (van Wyk *et al*., 2000; Watt and Breyer-Brandwijk, 1962; PP Ndlovu, personal communication).

Phytochemistry
Leonotis species contain several diterpenoids (labdane type lactones) such as marrubiin.

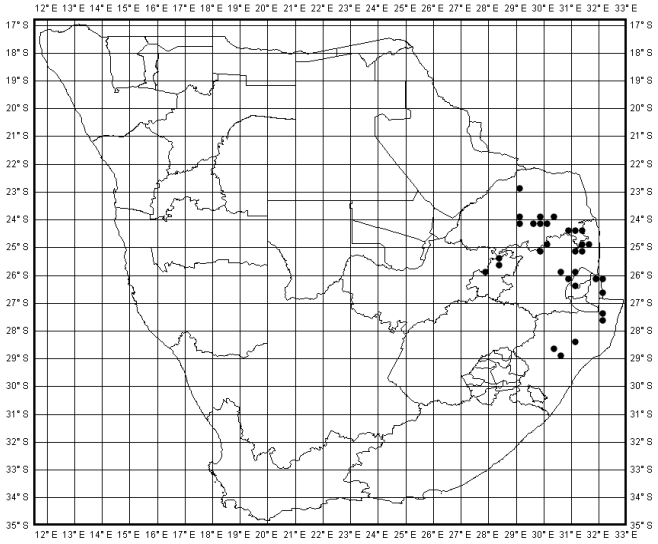
Pharmacological properties of *L. intermedia*.

Pharmacological properties	Flower	Stem
3H- hypoxanthine incorporation assay (IC_{50} in µg/ml ± SD)	55.51 ± 7.11	25.98 ± 1.74
Red blood cell lysis	>100 (3.77 ± 0.27%)	>100 (4.05 ± 0.01%)
Human kidney epithelial cell toxicity	107.81 ± 10.78	97.19 ± 10.50
Staphylococcus aureus (MIC in mg/ml)	4.00	4.00
Staphylococcus epidermidis (MIC in mg/ml)	3.00	3.00
Escherichia coli (MIC in mg/ml)	3.00	1.50
Klebsiella pneumoniae (MIC in mg/ml)	1.00	1.50
Candida albicans (MIC in mg/ml)	0.25	3.00
DPPH free radical scavenging (IC_{50} in µg/ml ± SD)	>100	>100
Metal chelating activity (IC_{50} in µg/ml± SD)	>100	>100
Lipid peroxidation (percent/100 µg/ml)	31.68 ± 3.44	11.87 ± 1.75
Total phenolic content (mg GAE/g extract)	129.00 ± 1.57	179.00 ± 3.5
Flavonoid content (mg RE/g extract)	<0.010	4.13 ± 0.23

B9. *Ozoroa sphaerocarpa* R. Fern & A. Fern

Scientific name: *Ozoroa sphaerocarpa* R. Fern & A. Fern
Family: Anacardiaceae
Vernacular names: currant resin tree (English), *imfuce* (Swati)

Ozoroa sphaerocarpa



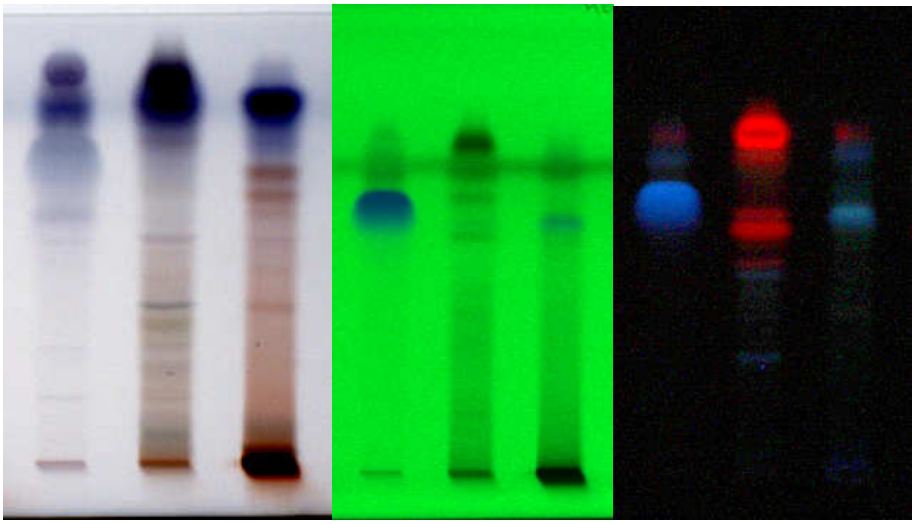
Ozoroa sphaerocarpa distribution map

Botanical description
It is a deciduous tree growing to a height of 12 m with a short, bent stem and sparsely spreading crown. The leaves are usually in whorls of three and the fruit is ellipsoid or kidney shaped, pale green at first, becoming black and wrinkled when ripe. It flowers between September and November (Adeniji *et al*., 2000; Watt and Breyer-Brandwijk, 1962).

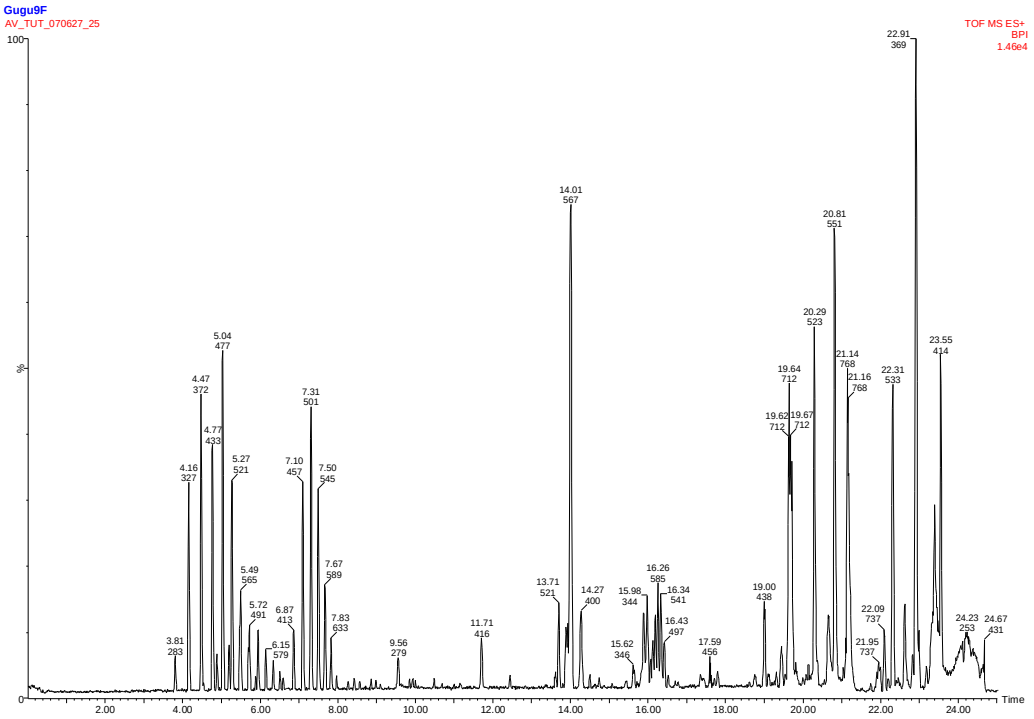
Distribution
The plant has wide distribution in deciduous woodland, bushveld and rocky hillsides.

Medicinal use
Medicinally, the bark is used for the treatment of diarrhoea (PP Ndlovu; personal communication). The leaf infusion is used together with *Athrixia phylicoides* to wash wounds (Adeniji *et al*., 2000).

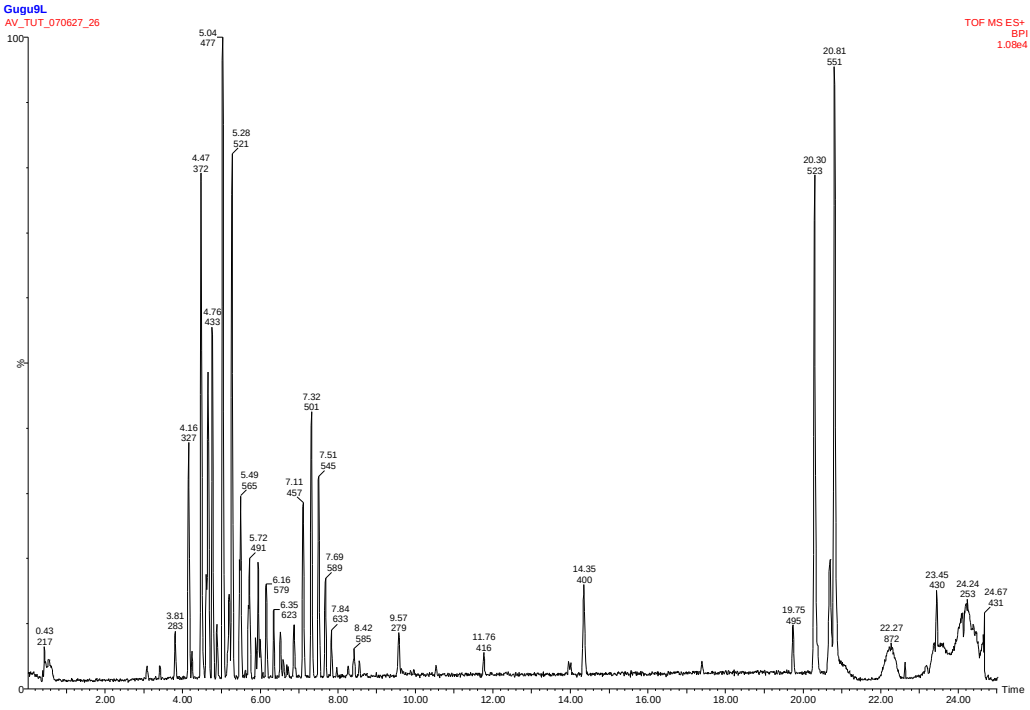
Phytochemistry



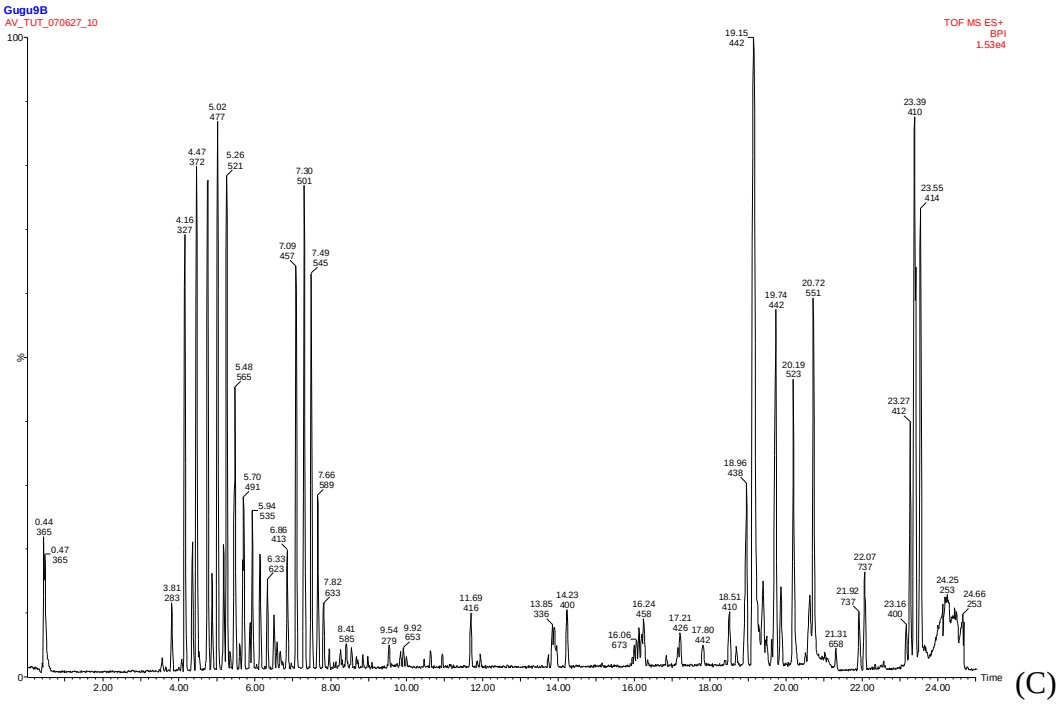
O. sphaerocarpa HPTLC chromatograms visualised after derivatization with vanillin-sulphuric acid and under 254 nm and 366 nm light, from left; fruit, leaves and bark.



(A)



(B)



(C)

Base peak intensity chromatograms for *O. sphaerocarpa* fruit (A), leaves (B)and bark (C).

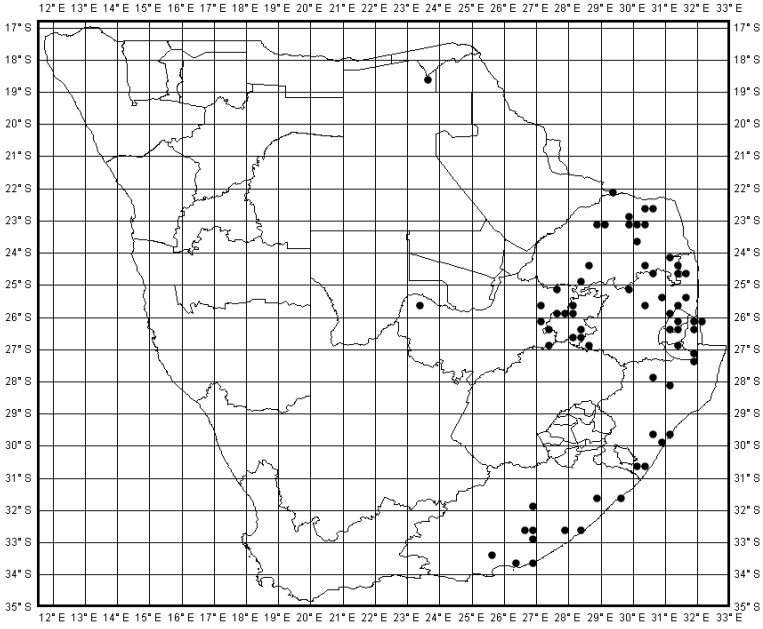
Pharmacological properties of *O. sphaerocarpa*.

Pharmacological properties	Fruit	Leaves	Bark
3H- hypoxanthine incorporation assay (IC_{50} in µg/ml ± SD)	40.20 ± 8.54	54.33 ± 1.24	45.29 ± 2.33
Red blood cell lysis	>100 (1.416 ± 0.02%)	180.26 ± 9.69	>100 (30.80 ± 5.55%)
Human kidney epithelial cell toxicity	10.39 ± 1.05	95.86 ± 26.27	8.11 ± 2.80
Staphylococcus aureus (MIC in mg/ml)	0.02	2.67	1.00
Staphylococcus epidermidis (MIC in mg/ml)	0.001	0.67	0.04
Escherichia coli (MIC in mg/ml)	4.00	2.67	1.00
Klebsiella pneumoniae (MIC in mg/ml)	0.07	0.50	0.67
Candida albicans (MIC in mg/ml)	0.58	1.00	1.00
DPPH free radical scavenging (IC_{50} in µg/ml ± SD)	11.63 ± 0.79	34.44 ± 4.08	11.23 ± 0.82
Metal chelating activity (IC_{50} in µg/ml± SD)	40.59 ± 3.52	58.72 ± 4.01	67.87 ± 4.08
Lipid peroxidation (percent/100 µg/ml)	76.47 ± 2.66	43.90 ± 1.09	80.10 ± 2.21
Total phenolic content (mg GAE/g extract)	<0.010	34.67 ± 2.25	<0.010
Flavonoid content (mg RE/g extract)	31.13 ± 1.10	1.30 ± 0.10	<0.010

B10. *Priva meyeri* Jaub and Spach var *meyeri*

Scientific name: *Priva meyeri* Jaub and Spach var *meyeri*
Family: **Verbenaceae**
Vernacular names: Sanama (Swati)

Priva meyeri



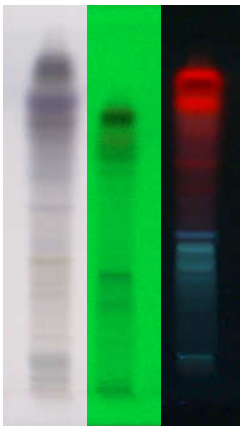
Priva meyeri distribution map

Botanical description
This is a herbaceous plant, becoming woody at the base and thinly rough-hairy throughout. Its leaves are opposite or scattered and the leaf stalk is about 1.3 cm long. The blade is triangular and broadest just above the base. Its inflorescence is terminal, elongated and indefinite. The followers are well spaced in axils of minute bracts. The corolla is mauve or white and five-lobed (Compton, 1976).

Distribution
The plant is distributed in the eastern part of Africa, from Tanzania in the north to Eastern Cape, South Africa in the south.

Medicinal use
The leaf infusion is traditionally used for sores and as ear drops for otitis media (PP Ndlovu; personal communication)(Watt and Breyer-Brandwijk, 1962).

Phytochemistry
Seeds contain tannins (Watt and Breyer-Brandwijk, 1962)



P. meyeri (whole plant) HPTLC chromatograms visualised after derivatization with vanillin-sulphuric acid and under 254 nm and 366 nm light.

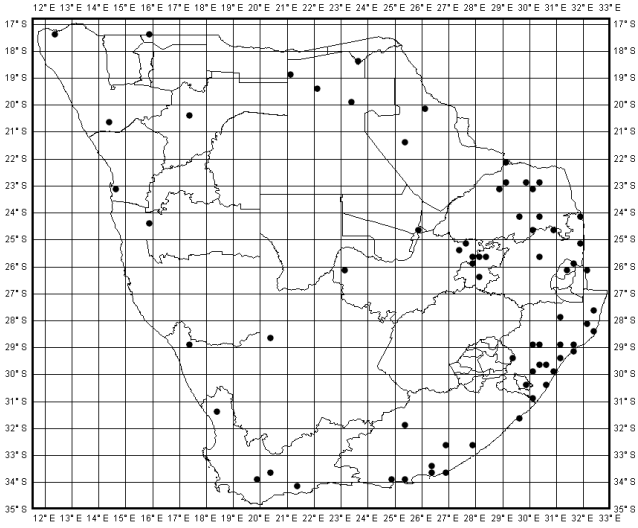
Pharmacological properties of *P. mayeri*.

Pharmacological properties	Whole plant
3H- incorporation assay (IC_{50} in µg/ml ± SD)	7.36 ± 0.85
Red blood cell lysis	>100 (11.107 ± 0.77)
Human kidney epithelial cell toxicity	14.63 ± 1.62
Staphylococcus aureus (MIC in mg/ml)	0.50
Staphylococcus epidermidis (MIC in mg/ml)	0.83
Escherichia coli (MIC in mg/ml)	0.50
Klebsiella pneumoniae (MIC in mg/ml)	1.33
Candida albicans (MIC in mg/ml)	0.67
DPPH free radical scavenging (IC_{50} in µg/ml ± SD)	70.04 ± 7.27
Metal chelating activity (IC_{50} in µg/ml± SD)	>100
Lipid peroxidation (percent/100 µg/ml)	84.37 ± 1.89
Total phenolic content (mg GAE/g extract)	295.43 ± 32.60
Flavonoid content (mg RE/g extract)	7.33 ± 1.16

B11. *Ricinus communis* L.

Scientific name: *Ricinus communis* L.
Family: Euphorbiaceae
Vernacular names: castor oil plant (English), *umhlafutfo* (Swati)

Ricinus communis leaf



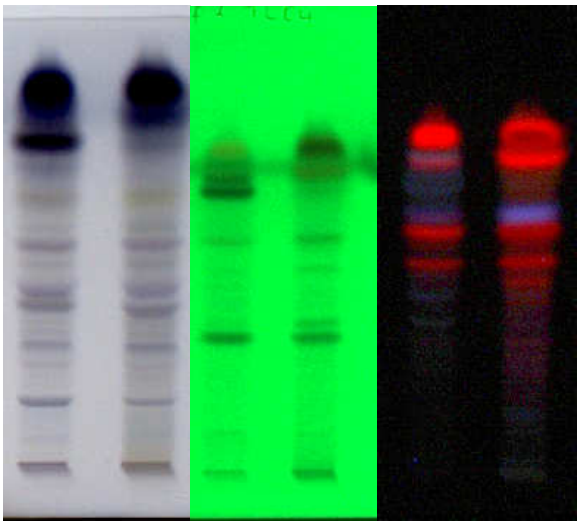
Ricinus communis distribution map

Botanical description
This is a large shrub of up to 4 m in height with very large, hand-shaped leaves on long, stout leaf stalks. The flower clusters appear near the tip of the branches. The fruits are three-lobed capsules with spine-like projections on their surfaces (Watt and Breyer-Brandwijk, 1962).

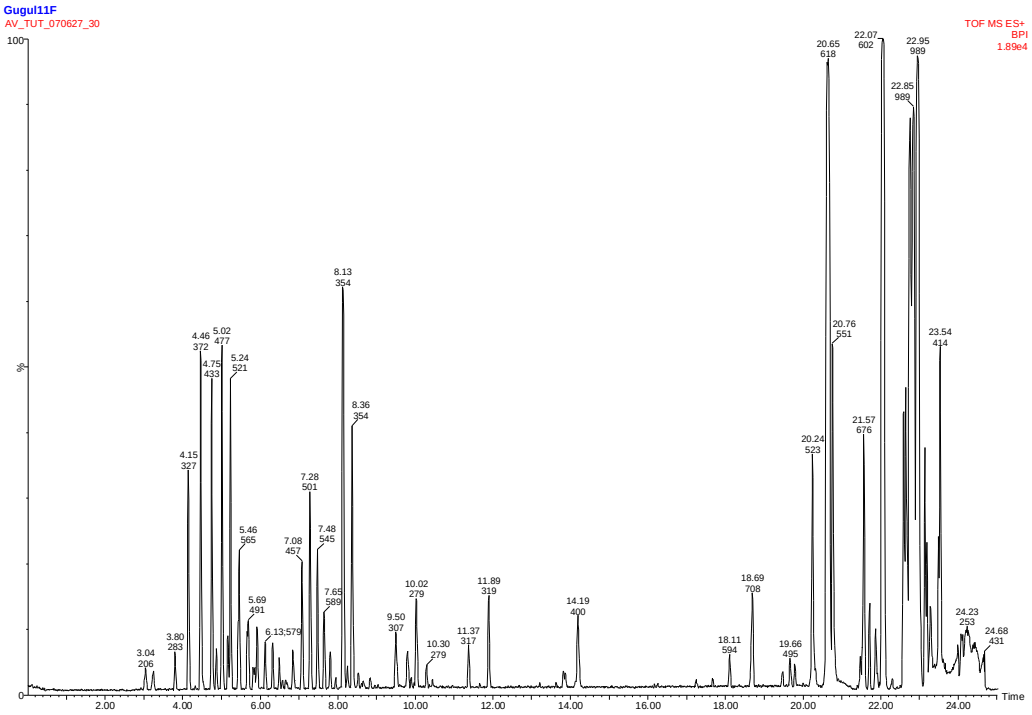
Distribution
This plant is exotic and believed to be indigenous to north-east Africa and India, but it has a wide distribution in tropical areas. It occurs predominantly as a weed on previously disturbed lands.

Medicinal use
The plant leaf infusion is medicinally administered orally or as an enema for the treatment of sores, while the unbroken seed is used for stomach-ache, toothache, sores, boils in children, headache, rheumatism and as a purgative (Watt and Breyer-Brandwijk, 1962; van Wyk *et al*., 2000).

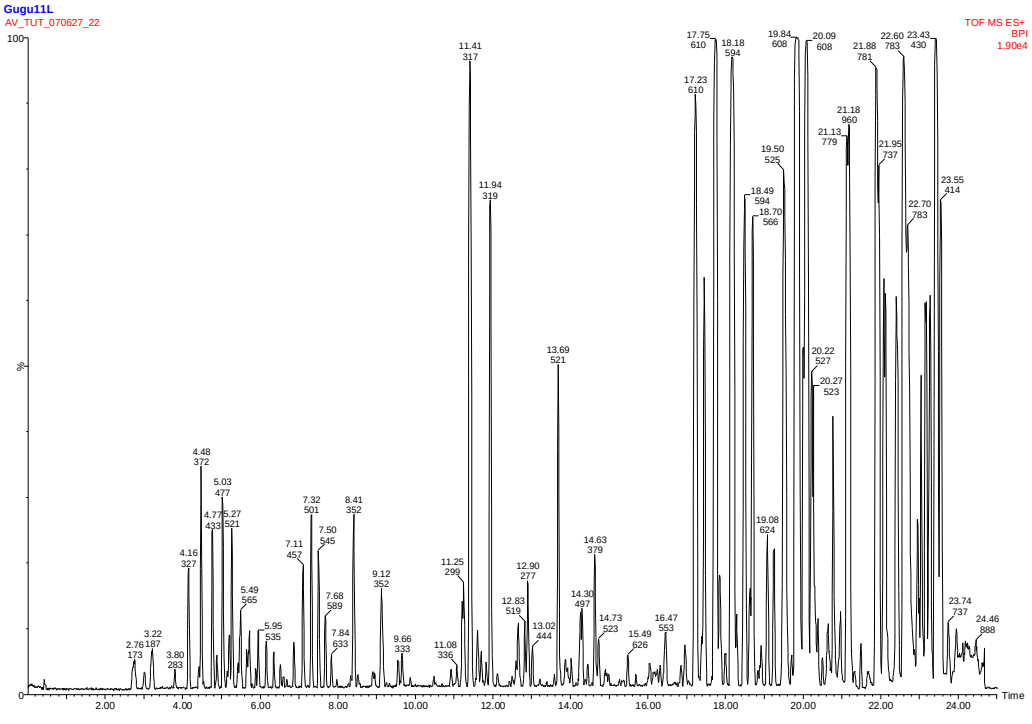
Phytochemistry
Castor oil contains a triglyceride fatty acid, seeds contain two highly toxic substances not present in the oil, an alkaloid: ricinine and a lectin: ricin (Wyk *et al*., 2000). Also has strong anti-oxidant activity due to the presence of flavonoids and tannins, anti-inflammatory (Ilavarasan *et al*., 2006) and antibacterial activity (Khan *et al*., 1978; Verpoorte and Dihal, 1987). Diterpene, caspene and mevalonic acid have been isolated from the seedlings (Sitton and West, 1975).



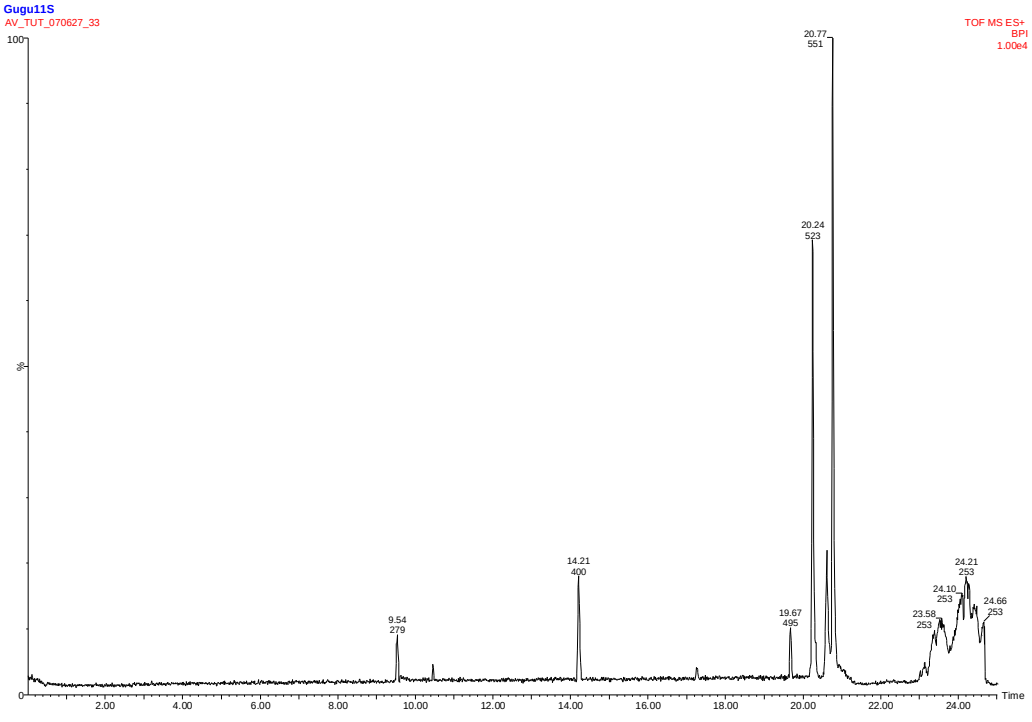
R. communis HPTLC chromatograms visualised after derivatization with vanillin-sulphuric acid and under 254 nm and 366 nm light, from left; fruit and leaves.



(A)



(B)



(C)

Base peak intensity chromatograms for *R. communis*; fruit (A), leaves (B) and stem (C).

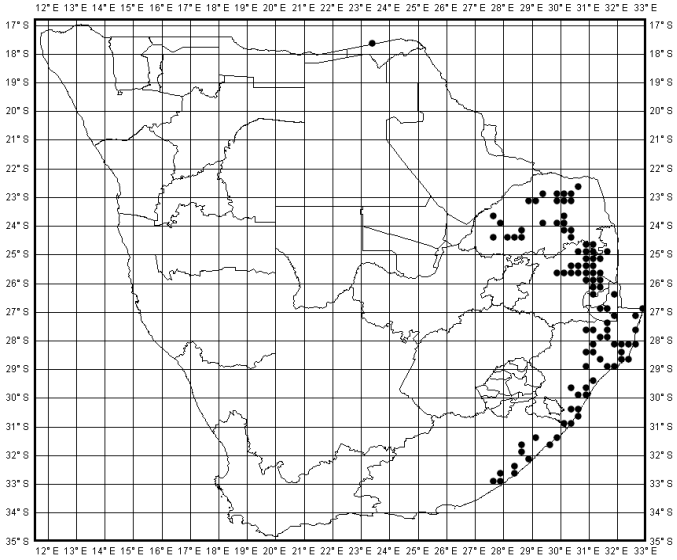
Pharmacological properties of *R. communis*.

Pharmacological properties	Fruit	Leaves	Stem
3H- hypoxanthine incorporation assay (IC_{50} in µg/ml ± SD)	33.61 ± 2.86	23.60 ± 2.28	52.73 ± 0.44
Red blood cell lysis	>100 (12.183 ± 0.18%)	>100 (13.01 ± 4.11%)	>100 (5.04 ± 0.17%)
Human kidney epithelial cell toxicity	136.11 ± 3.96	159.06 ± 5.00	137.53 ± 28.06
Staphylococcus aureus (MIC in mg/ml)	0.67	0.66	2.00
Staphylococcus epidermidis (MIC in mg/ml)	1.00	0.83	1.50
Escherichia coli (MIC in mg/ml)	1.33	1.33	1.50
Klebsiella pneumoniae (MIC in mg/ml)	1.33	3.33	1.00
Candida albicans (MIC in mg/ml)	0.50	0.29	0.50
DPPH free radical scavenging (IC_{50} in µg/ml ± SD)	>100	>100	>100
Metal chelating activity (IC_{50} in µg/ml± SD)	>100	32.80 ± 1.51	>100
Lipid peroxidation (percent/100 µg/ml)	63.74 ± 4.39	88.46 ± 3.25	16.98 ± 2.53
Total phenolic content (mg GAE/g extract)	263.37 ± 1.12	469.00 ± 55.72	197.00 ± 0.44
Flavonoid content (mg RE/g extract)	<0.010	4.07 ± 0.23	<0.010

B12. *Syzygium cordatum* Hochst ex C Krauss

Scientific name: *Syzygium cordatum* Hochst ex C Krauss
Family: Myrtaceae
Vernacular names: water berry (English), *umncozi* (Swati)

Syzygium cordatum



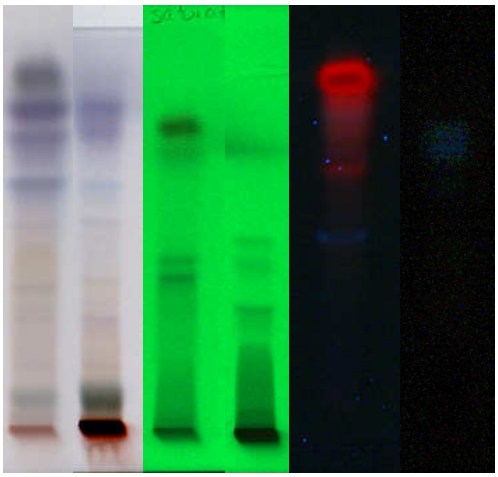
Syzygium cordatum distribution map

Botanical discription
The tree is evergreen and grows up to 20 m in height. The leaves are near ends of branches and broad, sometimes almost circular with a bluish-green colour. The flowers are cream to pink in colour and appear between August and November. The fruit is a berry that is egg-shaped and red to dark purple in colour. Its wood is used for boat-building, whilst the ripe fruit is edible (Venter and Venter, 2002; van Wyk *et al*., 2000).

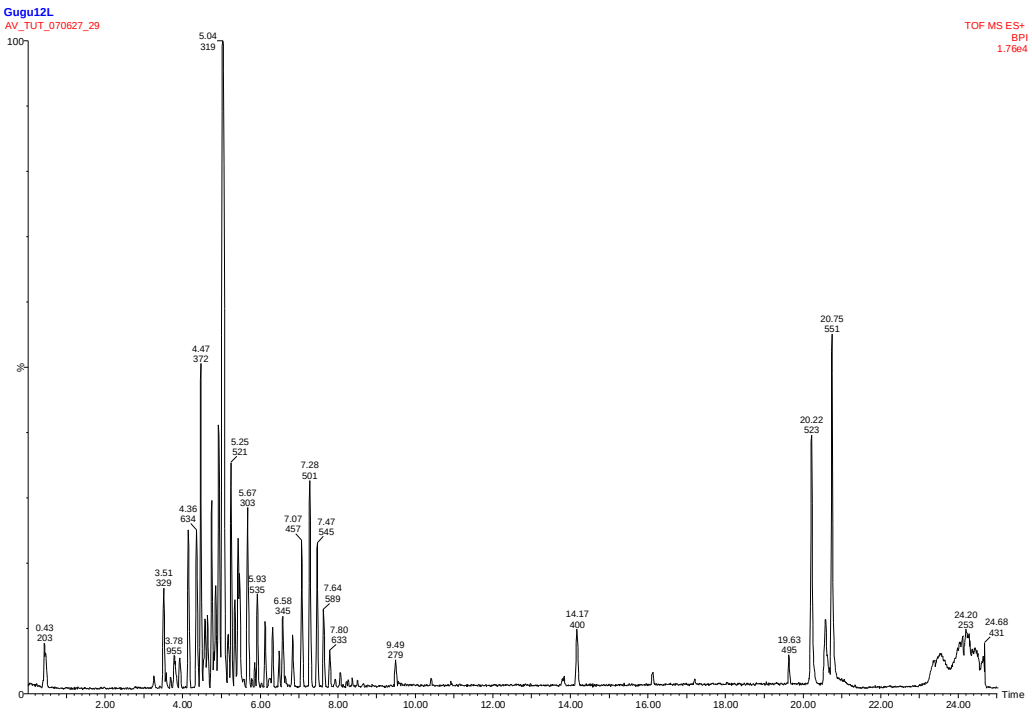
Distribution
The plant is distributed from Kenya to Eastern Cape in the south. In South Africa, it grows in the eastern and north-eastern parts and found along streams in riverine bush and forest.

Medicinal use
Medicinally the bark is used for diarrhoea, while the leaf, root and bark are used as an emetic, for the treatment of stomach troubles and respiratory ailments, including tuberculosis (Venter and Venter, 2002; van Wyk *et al.*, 2000).

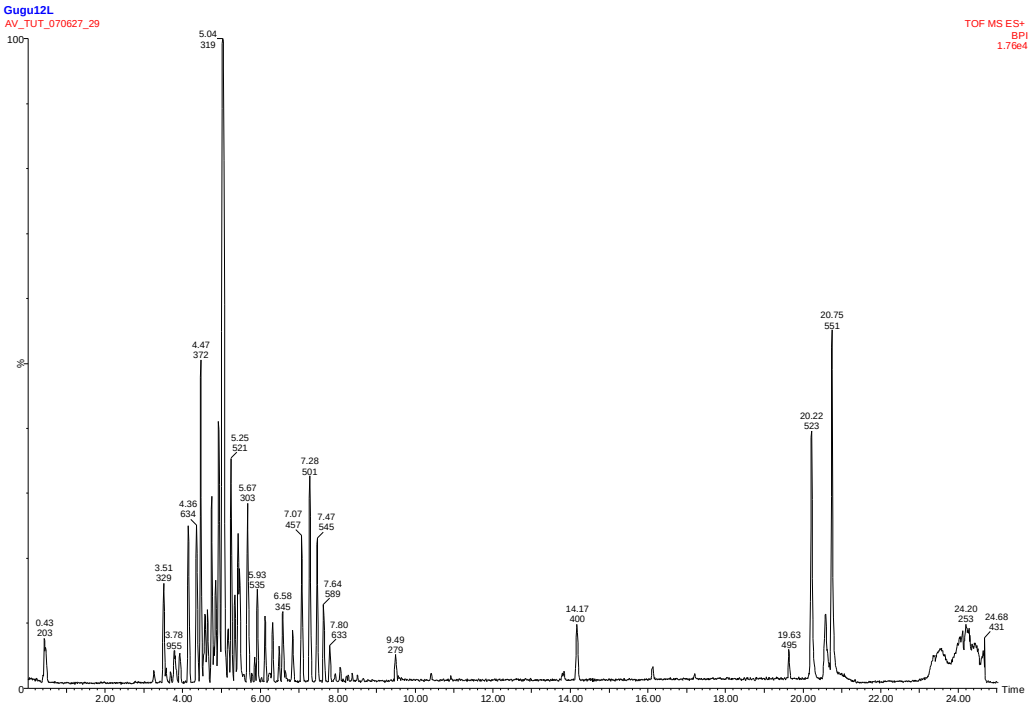
Phytochemistry
The wood and bark contain proanthocyanidins, pentacyclic triterpenoids, such as arjunolic acid, friedelin and epifriedelinol, cyaniding, delphidin, steroidal triterpenoids such as β-sitosterol, as well as gallic acid, ellagic acid and various gallic acid derivatives (Candy *et al*., 1968; Venter and Venter, 2002; van Wyk *et al.*, 2000).



S. cordatum HPTLC chromatograms visualised after derivatization with vanillin-sulphuric acid and under 254 nm and 366 nm light, from left; leaves and bark



(A)



(B)

Base peak intensity chromatograms for *S. cordatum* leaves (A) and bark (B).

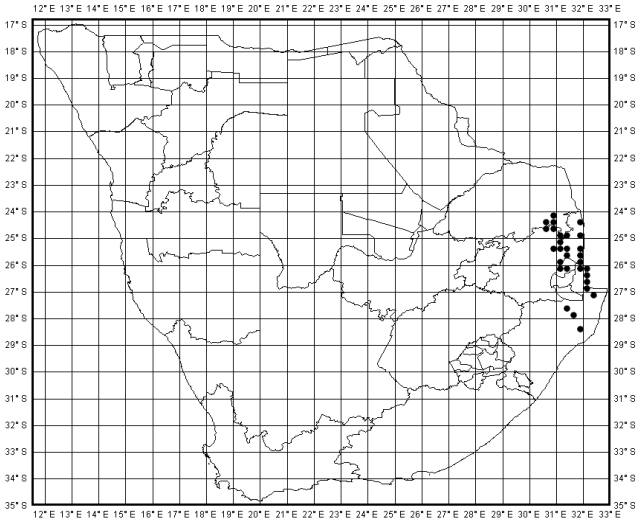
Pharmacological properties of *S. cordatum*.

Pharmacological properties	Leaves	Bark
3H- hypoxanthine incorporation assay (IC_{50} in µg/ml ± SD)	32.61 ± 2.05	6.70 ± 1.21
Red blood cell lysis	>100 (4.31 ± 0.41%)	>100 (22.83 ± 3.56%)
Human kidney epithelial cell toxicity	8.65 ± 1.41	26.80 ± 2.54
Staphylococcus aureus (MIC in mg/ml)	5.00	2.00
Staphylococcus epidermidis (MIC in mg/ml)	0.04	0.38
Escherichia coli (MIC in mg/ml)	3.00	1.50
Klebsiella pneumoniae (MIC in mg/ml)	0.44	0.25
Candida albicans (MIC in mg/ml)	1.00	1.50
DPPH free radical scavenging (IC_{50} in µg/ml ± SD)	15.76 ± 2.37	9.54 ± 0.92
Metal chelating activity (IC_{50} in µg/ml± SD)	>100	16.23 ± 0.99
Lipid peroxidation (percent/100 µg/ml)	73.07 ± 3.90	82.33 ± 2.21
Total phenolic content (mg GAE/g extract)	806.10 ± 42.28	33.70 ± 1.13
Flavonoid content (mg RE/g extract)	4.10 ± 0.78	27.13 ± 2.39

B13. *Terminalia phanerophlebia* Eng. & Diels

Scientific name: *Terminalia phanerophlebia* Eng. & Diels
Family: Combretaceae
Vernacular names: silver leaf tree (English), *emangwe lamnyama* (Swati)

Terminalia phanerophlebia flower



Terminalia phanerophlebia distribution map

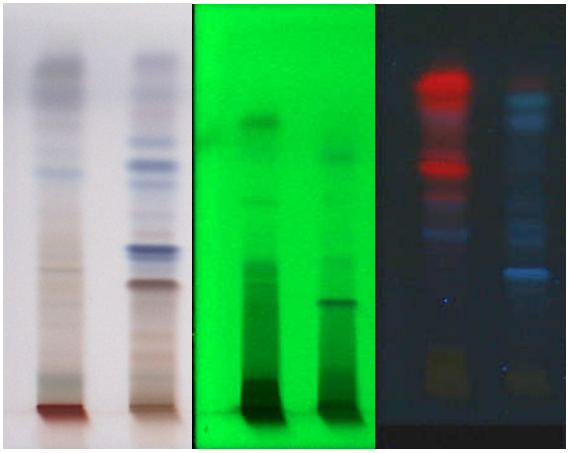
Botanical description
This is a small to medium-sized tree of about 5-10 m in height with an erect trunk and wide spreading crown. The silver-grey leaves are clustered at the tops of the branchlets, erect and more or less hairy when mature. The flowers are small and yellowish, with an unpleasant smell, and the fruit is pendulous (Neuwinger, 1996; van Wyk *et al*., 2000).

Distribution
The plant has a small distribution in northern Mpumalanga, South African and grows in Savannah lands on sandy soils.

Medicinal use
The bark infusion is drunk for the treatment of body pains and pneumonia. The root extract or decoction of many *Terminalia* species is used for diabetes, stomach problems, schistosomiasis, gonorrhoea, syphilis, diarrhoea, general body weakness, sore throat, colic and skin disease (Neuwinger, 1996; van Wyk *et al*., 2000).

Phytochemistry

Terminalia species are rich in triterpene saponins and tannins. Sometimes ellagic acid, and its methyl derivatives, quercetin and β-sitosterol are found (Neuwinger, 1996; van Wyk *et al*., 2000).



T. phanerophlebia HPTLC chromatograms visualised after derivatization with vanillin-sulphuric acid and under 254 nm and 366 nm light, from left; leaves and bark.

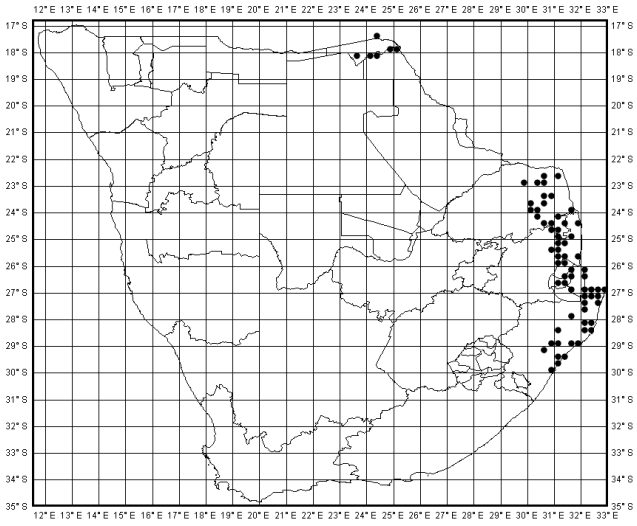
Pharmacological properties of *T. phanerophlebia*.

Pharmacological properties	Leaves	Bark
3H- hypoxanthine incorporation assay (IC_{50} in µg/ml ± SD)	7.81 ± 1.82	72.62 ± 3.40
Red blood cell lysis	>100(5.32 ± 1.43%)	>100 (5.25 ± 2.46%)
Human kidney epithelial cell toxicity	125.64 ± 13.99	>200 (68.98 ± 4.30%)
Staphylococcus aureus (MIC in mg/ml)	0.33	1.00
Staphylococcus epidermidis (MIC in mg/ml)	0.33	1.50
Escherichia coli (MIC in mg/ml)	1.33	1.00
Klebsiella pneumoniae (MIC in mg/ml)	0.67	1.33
Candida albicans (MIC in mg/ml)	0.77	0.75
DPPH free radical scavenging (IC_{50} in µg/ml ± SD)	7.09 ± 0.78	12.37 ± 3.03
Metal chelating activity (IC_{50} in µg/ml± SD)	14.43 ± 1.32	22.72 ± 1.33
Lipid peroxidation (percent/100 µg/ml)	91.63 ± 1.16	84.27 ± 0.49
Total phenolic content (mg GAE/g extract)	184.70 ± 2.19	174.70 ± 11.73
Flavonoid content (mg RE/g extract)	5.20 ± 0.20	33.33 ± 4.43

B14. *Trichilia emetica* Vahl

Scientific name: *Trichilia emetica* Vahl
Family: Meliaceae
Vernacular names: natal mahogany (English), *umkhuhlu* (Swati)

Trichilia emetica leaves



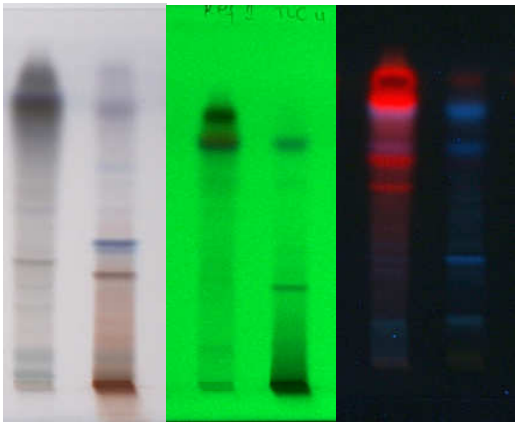
Trichilia emetica distribution map

Botanical description
It is a large densely leafy evergreen tree, growing to a height of about 20 m. The leaves are hairless above and thinly hairy below. The fruit is shortly stalked, round and downy (Venter and Venter, 2002; Watt and Breyer-Brandwijk, 1962).

Distribution
This plant has a wide distribution from Sudan to KwaZulu-Natal, South Africa in the south and grows in coastal, riverine and gallery forests.

Medicinal use
The bark is soaked in warm water and used to treat joint pains, nausea and vomiting, malaria, dysentery, fever, haematuria, urethral discharge and abdominal pain; whilst the bark or leaf is administered as an enema to treat a sore back. The seed oil is used to treat rectal ulceration and rheumatism, and the leaf or fruit poultice is used for bruises and eczema (Venter and Venter, 2002; Watt and Breyer-Brandwijk, 1962; Geyid *et al*., 2005: Adeniji *et al*., 2000; van Wyk *et al*., 2000).

Phytochemistry
Limonoids such as trichilin A and dregeanin have been isolated from the seed oil and are known for their antimicrobial and anti-inflammatory activity (van Wyk *et al*., 2000). Others are palmitic acid, oleic acid and tannins (Watt and Breyer-Brandwijk, 1962). Also polyphenols such as caffeic acid, ferulic acid, gallic acid, chlorogenic acid and syringic acid (Germanó *et al*., 2006) alkaloids, flavonoids, glycosides, and steroids (Amusan *et al*., 2007).



T. emetica HPTLC chromatograms visualised after derivatization with vanillin-sulphuric acid and under 254 nm and 366 nm light, from left; leaves and bark.

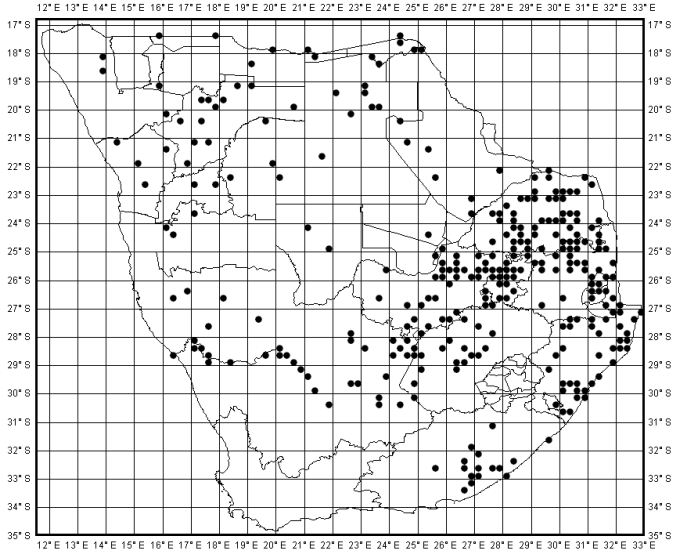
Pharmacological properties of *T. emetica*.

Pharmacological properties	Leaves	Bark
3H- hypoxanthine incorporation assay (IC_{50} in µg/ml ± SD)	18.13 ± 3.55	>200 (71.07 ± 7.78%)
Red blood cell lysis	>100 (6.25 ± 0.68%)	194.38 ± 7.25
Human kidney epithelial cell toxicity	45.95 ± 5.10	67.83 ± 2.78
Staphylococcus aureus (MIC in mg/ml)	0.19	0.58
Staphylococcus epidermidis (MIC in mg/ml)	0.16	0.07
Escherichia coli (MIC in mg/ml)	0.83	6.67
Klebsiella pneumoniae (MIC in mg/ml)	2.00	2.67
Candida albicans (MIC in mg/ml)	0.22	0.78
DPPH free radical scavenging (IC_{50} in µg/ml ± SD)	70.59 ± 12.68	15.16 ± 1.75
Metal chelating activity (IC_{50} in µg/ml± SD)	44.21 ± 4.25	>100
Lipid peroxidation (percent/100 µg/ml)	59.05 ± 1.83	59.73 ± 2.59
Total phenolic content (mg GAE/g extract)	329.00 ± 5.92	17.00 ± 1.89
Flavonoid content (mg RE/g extract)	<0.010	26.40 ± 0.72

B15. *Ziziphus mucronata* Willd

Scientific name: *Ziziphus mucronata* Willd
Family: Rhamnaceae
Vernacular names: buffalo thorn (English), *umlahlabantfu* (Swati)

Ziziphus mucronata



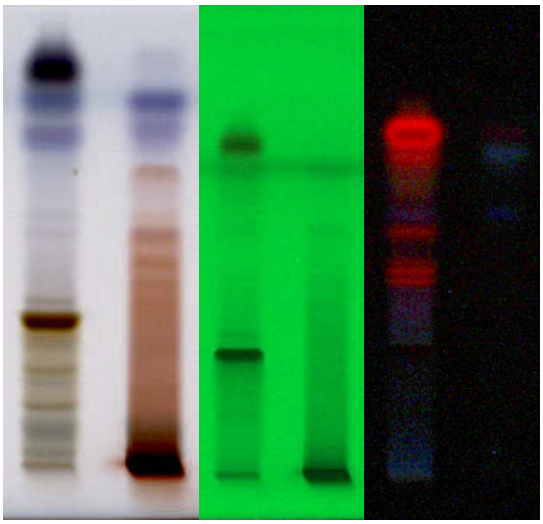
Ziziphus mucronata distribution map

Botanical description
This deciduous tree can grow up to a height of 17 m and has an open round to spreading crown. The leaves are alternate, simple, smooth and shiny. The flowers are clusters in the leaf axils, yellowish green in colour with short flower stalks, and appear between October and April. The fruit is a round, reddish brown glossy drupe (Venter and Venter, 2002; Neuwinger, 1996; Watt and Breyer-Brandwijk, 1962).

Distribution
It has a wide distribution in Africa, from Arabia to Western Cape, South Africa in the south and grows mostly in woodland and wooded grassland.

Medicinal use
The bark powder is used for body pains, and cough, while the leaf poultice is used for the treatment of chest troubles, boils and other septic swellings. The root infusion is used for treating dysentery, gonorrhoea and sores (Venter and Venter, 2002; Neuwinger, 1996; Watt and Breyer-Brandwijk, 1962).

Phytochemistry
The bark contains 12-15% tannin (Venter and Venter, 2002). *Ziziphus* species generally contains alkaloids, sterols/triterpenoids, saponins, tannins, flavonoids (Rauf *et al*., 1986).



Z. mucronata HPTLC chromatograms visualised after derivatization with vanillin-sulphuric acid and under 254 nm and 366 nm light, from left; leaves and bark.

Pharmacological properties of *Z. mucronata*.

Pharmacological properties	Fruit	Leaves	Bark
3H- hypoxanthine incorporation assay (IC_{50} in µg/ml ± SD)	67.18 ± 7.40	73.99 ± 1.47	94.84 ± 1.47
Red blood cell lysis	>100 (3.21 ± 0.09%)	>100 (4.37 ± 0.31%)	>100 (3.44 ± 0.81%)
Human kidney epithelial cell toxicity	>200 (55.68 ± 3.68%)	61.17 ± 1.78	2.23 ± 0.91
Staphylococcus aureus (MIC in mg/ml)	3.33	4.00	0.50
Staphylococcus epidermidis (MIC in mg/ml)	1.33	2.00	1.00
Escherichia coli (MIC in mg/ml)	4.00	3.00	2.00
Klebsiella pneumoniae (MIC in mg/ml)	1.33	1.50	1.33
Candida albicans (MIC in mg/ml)	4.00	0.23	4.00
DPPH free radical scavenging (IC_{50} in µg/ml ± SD)	43.54 ± 1.24	>100	15.27 ± 2.22
Metal chelating activity (IC_{50} in µg/ml± SD)	>100	>100	55.67 ± 1.71
Lipid peroxidation (percent/100 µg/ml)	26.43 ± 2.04	28.66 ± 1.18	38.73 ± 2.36
Total phenolic content (mg GAE/g extract)	536.87 ± 36.76	218.00 ± 5.76	46.27 ± 5.61
Flavonoid content (mg RE/g extract)	<0.010	3.40 ± 0.20	<0.010

APPENDIX C

Human Ethics Clearance Waiver

APPENDIX D: Sum Fractional Inhibitory Concentration for Malaria Combination

Table D1: Sum fractional inhibitory concentrations for *F. glumosa* leaves (Fg) with quinine, tested against *Plasmodium falciparum* obtained in experiment 1.

Concentrations (µg/ml)		IC_{50} Values				FIC		
Quinine	Fg	Quinine	s.d.	Fg	s.d.	FIC Quinine	FIC Fg	ΣFIC
0.3	0	0.02	0.01	0.00	-			
0.25	0.1	0.059	-	0.02	0.00	3.525	0.003	3.528
2	0.5	0.055	-	0.01	0.00	3.293	0.002	3.295
1	1	0.031	-	0.03	0.00	1.864	0.004	1.868
0.5	5	0.008	-	0.08	0.00	0.471	0.010	0.481
0.1	10	0.008	-	0.75	0.08	0.451	0.098	0.549
0.01	20	0.002	-	4.72	1.31	0.141	0.615	0.756
0	30	-	-	7.67	1.79		SUM	10.476
							AVG	1.746

Table D2: Sum fractional inhibitory concentrations for *F. glumosa* leaves (Fg) with quinine, tested against *Plasmodium falciparum* obtained in duplicate experiment 2.

Concentrations (µg/ml)		IC_{50} Values				FIC		
Quinine	Fg	Quinine	s.d.	Fg	s.d.	FIC Quinine	FIC Fg	ΣFIC
0.3	0	0.02	0.00	0.00	-			
0.25	0.1	0.040	-	0.02	0.00	2.285	0.003	2.287
2	0.5	0.061	-	0.02	0.00	3.448	0.003	3.450
1	1	0.039	-	0.04	0.00	2.207	0.007	2.214
0.5	5	0.007	-	0.07	0.00	0.410	0.012	0.422
0.1	10	0.009	-	0.87	0.09	0.493	0.146	0.639
0.01	20	0.002	-	3.92	0.47	0.111	0.660	0.771
0	30	-	-	5.95	0.44		SUM	9.785
							AVG	1.631

Overall ΣFIC = 1.688 ± 0.081

Table D3: Sum fractional inhibitory concentrations for *B. salicina* bark (Bs) with quinine, tested against *Plasmodium falciparum* obtained with experiment 1.

Concentrations (µg/ml)		IC_{50} Values				FIC		
Quinine	**Bs**	**Quinine**	**s.d.**	**Bs**	**s.d.**	**FIC Quinine**	**FIC Bs**	**ΣFIC**
0.3	0	0.01	0.00	0.00	-			
0.25	0.1	0.027	-	0.01	0.00	2.690	0.002	2.692
2	0.5	0.028	-	0.01	0.00	2.744	0.001	2.745
1	1	0.012	-	0.01	0.00	1.192	0.002	1.194
0.5	5	0.007	-	0.07	0.01	0.695	0.012	0.707
0.1	10	0.020	-	1.97	0.01	1.959	0.335	2.294
0.01	20	0.002	-	3.30	0.29	0.164	0.561	0.725
0	40	-	-	5.88	0.90		SUM	10.357
							AVG	1.726

Table D4: Sum fractional inhibitory concentrations for *B. salicina* bark (Bs) with quinine, tested against *Plasmodium falciparum* obtained with duplicate experiment 2.

Concentrations (µg/ml)		IC_{50} Values				FIC		
Quinine	**Bs**	**Quinine**	**s.d.**	**Bs**	**s.d.**	**FIC Quinine**	**FIC Bs**	**ΣFIC**
0.3	0	0.02	0.01	0.00	-			
0.25	0.1	0.045	-	0.02	0.00	2.844	0.002	2.846
2	0.5	0.037	-	0.01	0.00	2.284	0.001	2.285
1	1	0.026	-	0.03	0.00	1.613	0.002	1.615
0.5	5	0.025	-	0.25	0.00	1.533	0.023	1.557
0.1	10	0.029	-	2.87	0.07	1.795	0.274	2.069
0.01	20	0.002	-	3.19	0.03	0.099	0.305	0.404
0	40	-	-	10.47	0.10		SUM	10.776
							AVG	1.796

Overall ΣFIC = 1.761 ± 0.049

Table D5: Sum fractional inhibitory concentrations for *L. intermedia* stem (Li) with quinine, tested against *Plasmodium falciparum* obtained in experiment 1.

Concentrations (µg/ml)		IC_{50} Values				FIC		
Quinine	**Li**	**Quinine**	**s.d.**	**Li**	**s.d.**	**FIC Quinine**	**FIC Li**	**ΣFIC**
0.3	0	0.01	0.01	0.00	-			
0.25	0.1	0.034	-	0.01	0.00	3.423	0.001	3.424
2	0.5	0.024	-	0.01	0.01	2.335	0.0004	2.336
1	1	0.016	-	0.02	0.01	1.632	0.001	1.633
0.5	5	0.020	-	0.20	0.02	1.959	0.016	1.975
0.1	10	0.015	-	1.54	0.22	1.528	0.122	1.650
0.01	20	0.005	-	9.25	0.81	0.459	0.736	1.196
0	40	-	-	12.56	3.01		SUM	12.213
							AVG	2.036

Table D6: Sum fractional inhibitory concentrations for *L. intermedia* stem (Li) with quinine, tested against *Plasmodium falciparum* obtained in duplicate experiment 2.

Concentrations (µg/ml)		IC_{50} Values				FIC		
Quinine	**Li**	**Quinine**	**s.d.**	**Li**	**s.d.**	**FIC Quinine**	**FIC Li**	**ΣFIC**
0.3	0	0.01	0.00	0.00	-			
0.25	0.1	0.040	-	0.02	0.00	3.1417	0.0006	3.142
2	0.5	0.070	-	0.02	0.01	5.552569	0.0007	5.553
1	1	0.015	-	0.01	0.01	1.173913	0.0006	1.174
0.5	5	0.022	-	0.22	0.00	1.721446	0.009	1.730
0.1	10	0.031	-	3.12	0.08	2.467154	0.125	2.592
0.01	20	0.006	-	11.04	0.98	0.43651	0.443	0.879
0	40	-	-	24.92	1.34		SUM	9.519
							AVG	1.904

Overall ΣFIC = 1.970 ± 0.093

Table D7: Sum fractional inhibitory concentrations for *T. phanerophlebia* leaves (Tp) with quinine, tested against *Plasmodium falciparum* obtained in experiment 1.

Concentrations (µg/ml)		IC_{50} Values				FIC		
Quinine	**Tp**	**Quinine**	**s.d.**	**Tp**	**s.d.**	**FIC Quinine**	**FIC Tp**	**ΣFIC**
0.3	0	0.02	0.00	0.00	-			
0.25	0.1	0.047	-	0.02	0.00	2.933	0.001	2.934
2	0.5	0.052	-	0.01	0.00	3.196	0.0006	3.197
1	1	0.035	-	0.04	0.00	2.187	0.002	2.189
0.5	5	0.016	-	0.16	0.02	1.005	0.008	1.013
0.1	10	0.050	-	5.02	0.05	3.112	0.259	3.372
0.01	20	0.004	-	7.70	1.20	0.239	0.398	0.637
0	30	-	-	19.36	1.26		SUM	13.342
							AVG	2.224

Table D8: Sum fractional inhibitory concentrations for *T. phanerophlebia* leaves (Tp) with quinine, tested against *Plasmodium falciparum* obtained in duplicate experiment 2.

Concentrations (µg/ml)		IC_{50} Values				FIC		
Quinine	**Tp**	**Quinine**	**s.d.**	**Tp**	**s.d.**	**FIC Quinine**	**FIC Tp**	**ΣFIC**
0.3	0	0.01	0.00	0.00	-			
0.25	0.1	0.034	-	0.01	0.00	2.339	0.002	2.340
2	0.5	0.027	-	0.01	0.00	1.839	0.001	1.840
1	1	0.013	-	0.01	0.00	0.884	0.002	0.886
0.5	5	0.008	-	0.08	0.02	0.513	0.010	0.523
0.1	10	0.046	-	4.60	0.20	3.133	0.645	3.779
0.01	20	0.005	-	10.21	0.33	0.348	1.433	1.781
0	30	-	-	7.13	0.34		SUM	9.368
							AVG	1.874

Overall ΣFIC = 2.049 ± 0.248

Table D9: Sum fractional inhibitory concentrations for *B. setifera* stem/root (Bs) with quinine, tested against *Plasmodium falciparum* obtained in experiment 1.

Concentrations (µg/ml)		IC_{50} Values				FIC		
Quinine	**Bs**	**Quinine**	**s.d.**	**Bs**	**s.d.**	**FIC Quinine**	**FIC Bs**	**ΣFIC**
0.3	0	0.01	0.00	0.00	-			
0.25	0.1	0.045	-	0.02	0.00	5.242	0.002	5.244
2	0.5	0.155	-	0.04	0.00	18.142	0.004	18.146
1	1	0.017	-	0.02	0.00	1.980	0.002	1.982
0.5	5	0.015	-	0.15	0.06	1.814	0.016	1.830
0.1	10	0.022	-	2.23	0.41	2.616	0.235	2.852
0.01	20	0.005	-	10.00	0.47	0.585	1.054	1.639
0	30	-	-	9.49	0.04		SUM	31.693
							AVG	5.282

Table D10: Sum fractional inhibitory concentrations for *B. setifera* stem/root (Bs) with quinine, tested against *Plasmodium falciparum* obtained in duplicate experiment 2.

Concentrations (µg/ml)		IC_{50} Values				FIC		
Quinine	**Bs**	**Quinine**	**s.d.**	**Bs**	**s.d.**	**FIC Quinine**	**FIC Bs**	**ΣFIC**
0.3	0	0.01	0.00	0.00	-			
0.25	0.1	0.035	-	0.01	0.00	4.156	0.001	4.157
2	0.5	0.100	-	0.02	0.00	11.702	0.002	11.704
1	1	0.059	-	0.06	0.00	6.933	0.005	6.939
0.5	5	0.046	-	0.46	0.00	5.431	0.043	5.473
0.1	10	0.041	-	4.08	0.05	4.781	0.375	5.156
0.01	20	0.004	-	7.48	0.65	0.438	0.686	1.124
0	30	-	-	10.90	0.60		SUM	34.553
							AVG	5.759

Overall ΣFIC = 5.521 ± 0.337

APPENDIX E: WAVELENGTH SCAN FOR DETERMINING ABSORBANCE MAXIMA TO BE USED FOR ABSORBANCE MEASUREMENTS.

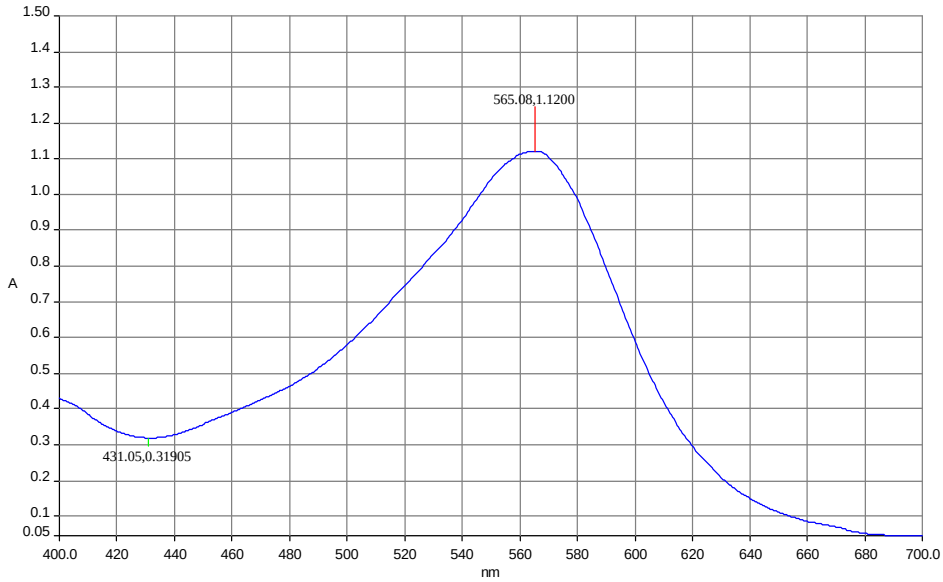


Figure E1: Wavelength scan for the absorbance maxima of the ferrozine-iron complex between 400 and 700 nm, with maxima at 565.08 nm.

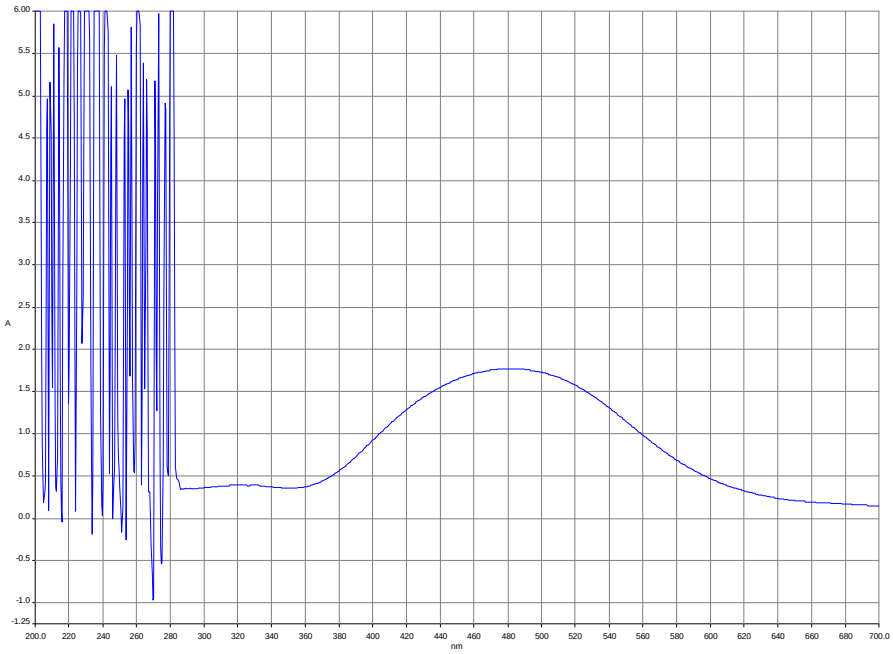


Figure E2: Wavelength scan for the absorbance maxima of the ferric ions–thiocynate complex between 200 and 700 nm, with maxima at 481.04 nm.

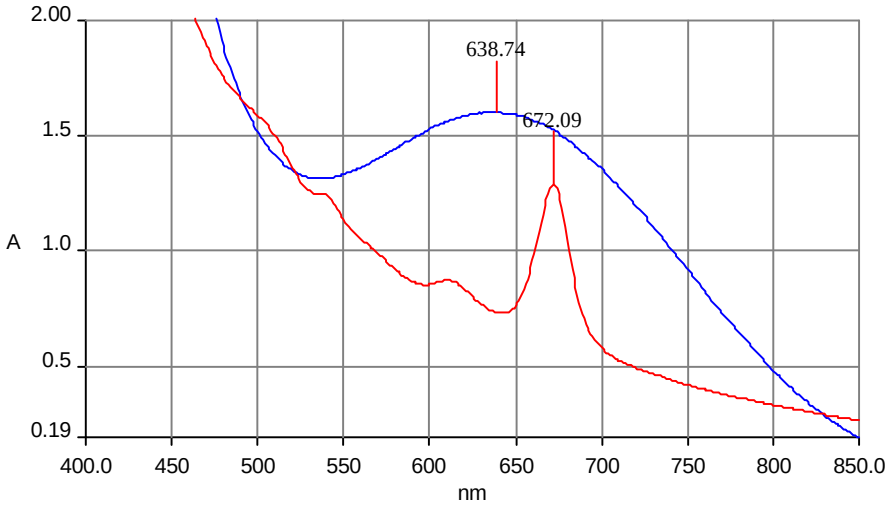


Figure E3: Wavelength scan for the absorbance maxima of the gallic acid and Folin-Ciocalteu reagent complex (blue) and *O. sphaerocarpa* leaves (red), measured between 400 and 850 nm, with maxima at 638.74 and 672.09 nm, respectively.

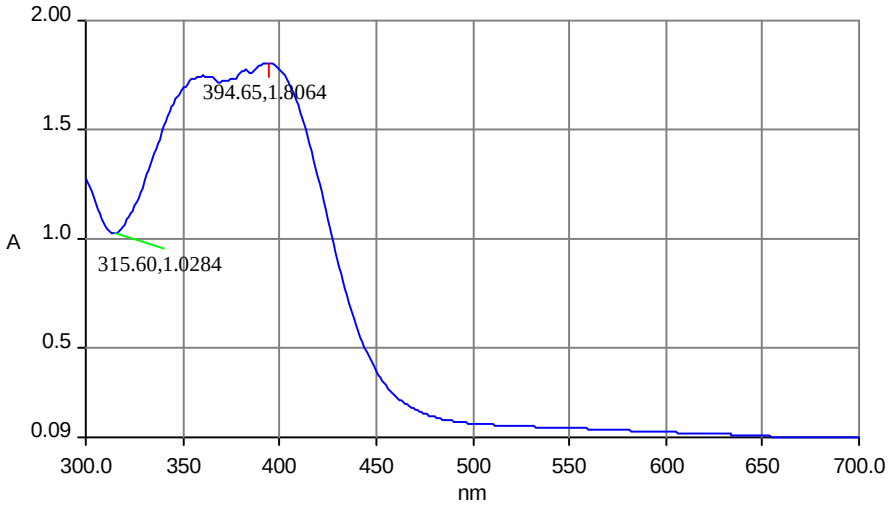


Figure E4: Wavelength scan for the absorbance maxima of the rutin-aluminium complex with maxima at 394.65 nm.

APPENDIX F: Sum Fractional Inhibitory Concentration for Two Plant Combination Against *E. coli*

Table F1: Chart used to determine the MIC values of the different plant combinations.

Ratio and volume of extract		Rows in microtitre plate							
		A	**B**	**C**	**D**	**E**	**F**	**G**	**H**
	Plant A	16	8	4	2	1	0.5	0.25	0.125
(100:0)	**Plant B**	0	0	0	0	0	0	0	0
	Plant A	14.4	7.2	3.6	1.8	0.9	0.45	0.225	0.1125
(90:10)	**Plant B**	1.6	0.8	0.4	0.2	0.1	0.05	0.025	0.0125
	Plant A	12.8	6.4	3.2	1.6	0.8	0.4	0.2	0.1
(80:20)	**Plant B**	3.2	1.6	1.6	0.8	0.4	0.2	0.1	0.05
	Plant A	11.2	5.6	2.8	1.4	0.7	0.35	0.175	0.0875
(70:30)	**Plant B**	4.8	2.4	1.2	0.6	0.3	0.15	0.075	0.0375
	Plant A	9.6	4.8	2.4	1.2	0.6	0.3	0.15	0.075
(60:40)	**Plant B**	6.4	3.2	1.6	0.8	0.4	0.2	0.1	0.05
	Plant A	8	4	2	1	0.5	0.25	0.125	0.0625
(50:50)	**Plant B**	8	4	2	1	0.5	0.25	0.125	0.0625
	Plant A	6.4	3.2	1.6	0.8	0.4	0.2	0.1	0.05
(40:60)	**Plant B**	9.6	4.8	2.4	1.2	0.6	0.3	0.15	0.075
	Plant A	4.8	2.4	1.2	0.6	0.3	0.15	0.075	0.0375
(30:70)	**Plant B**	11.2	5.6	2.8	1.4	0.7	0.35	0.175	0.0875
	Plant A	3.2	1.6	1.6	0.8	0.4	0.2	0.1	0.05
(20:80)	**Plant B**	12.8	6.4	3.2	1.6	0.8	0.4	0.2	0.1
	Plant A	1.6	0.8	0.4	0.2	0.1	0.05	0.025	0.0125
(10:90)	**Plant B**	14.4	7.2	3.6	1.8	0.9	0.45	0.225	0.1125
	Plant A	0	0	0	0	0	0	0	0
(0:100)	**Plant B**	16	8	4	2	1	0.5	0.25	0.125

Table F2: Sum fractional inhibitory concentrations for *B. salicina* bark with *S. cordatum* bark, tested against *E. coli*.

Concentrations (mg/ml)		ΣFIC		
***B. salicina* bark**	***S. cordatum* bark**	**Experiment 1**	**Experiment 2**	**Experiment 3**
16	0	1	1	1
14.4	1.6	0.65	0.65	2.6
12.8	3.2	0.8	0.8	0.8
11.2	4.8	0.475	0.475	0.95
9.6	6.4	0.55	0.55	0.55
8	8	0.625	0.625	0.625
6.4	9.6	0.7	0.7	0.7
4.8	11.2	0.775	0.775	0.775
3.2	12.8	0.85	0.85	0.85
1.6	14.4	0.925	0.925	0.925
0	16	1	1	1
			Average FIC	**0.728**
			s.d.	**0.038**

Table F3: Sum fractional inhibitory concentrations for *O. sphaerocarpa* bark with *S. cordatum* bark, tested against *E. coli*.

Concentrations (mg/ml)		ΣFIC		
***O. sphaerocarpa* bark**	***S. cordatum* bark**	**Experiment 1**	**Experiment 2**	**Experiment 3**
16	0	1	1	1
14.4	1.6	0.55	0.55	1.1
12.8	3.2	0.6	0.6	0.6
11.2	4.8	0.65	0.325	0.325
9.6	6.4	0.7	0.35	0.35
8	8	0.75	0.375	0.375
6.4	9.6	0.8	0.4	0.4
4.8	11.2	0.85	0.425	0.425
3.2	12.8	0.9	0.45	0.45
1.6	14.4	0.95	0.475	0.475
0	16	1	1	1
			Average FIC	**0.563**
			s.d.	**0.165**

Table F4: Sum fractional inhibitory concentrations for *O. sphaerocarpa* bark with *B. salicina* bark, tested against *E. coli*.

Concentrations (mg/ml)		**ΣFIC**		
***O. sphaerocarpa* bark**	***B. salicina* bark**	**Experiment 1**	**Experiment 2**	**Experiment 3**
16	0	1	1	1
14.4	1.6	0.456	0.456	0.456
12.8	3.2	0.825	0.825	0.825
11.2	4.8	0.36875	0.737	0.737
9.6	6.4	0.65	0.325	0.65
8	8	1.125	1.125	0.5625
6.4	9.6	0.95	0.95	0.475
4.8	11.2	1.55	1.55	0.775
3.2	12.8	1.2	1.2	0.6
1.6	14.4	0.85	0.85	0.85
0	16	1	1	1
			Average FIC	**0.888**
			s.d.	**0.132**

APPENDIX G: Sum Fractional Inhibitory Concentration for Three Plant Combination Against *E. coli*

Table G1: Sum fractional inhibitory concentrations for *B. salicina* bark and *S. cordatum* bark with different concentrations of *O. sphaerocarpa*, tested against *E. coli*.

Concentrations (mg/ml)		**ΣFIC**			
***B. salicina* bark**	***S. cordatum* bark**	**Without *O. sphaerocarpa***	**½ MIC *O. sphaerocarpa***	**MIC *O. sphaerocarpa***	**2 X MIC *O. sphaerocarpa***
16	0	1	1	1	1
14.4	1.6	0.65	0.5	0.85	0.925
12.8	3.2	0.8	1	1.2	0.85
11.2	4.8	0.475	0.5	0.387	1.55
9.6	6.4	0.55	0.5	0.95	0.35
8	8	0.625	0.5	1.125	0.312
6.4	9.6	0.7	0.5	0.65	0.275
4.8	11.2	0.775	1	0.737	0.237
3.2	12.8	0.85	0.5	1.65	0.2
1.6	14.4	0.925	1	0.912	0.162
0	16	1	1	1	1
	Average FIC	**0.727662**	**0.666667**	**0.940278**	**0.540**

Table G2: Sum fractional inhibitory concentrations for *O. sphaerocarpa* bark and *S. cordatum* bark with different concentration of *B. salicina*, tested against *E. coli*.

Concentrations (mg/ml)		**ΣFIC**			
***O. sphaerocarpa* bark**	***S. cordatum* bark**	**Without *B. salicina***	**½ MIC *B. salicina***	**MIC *B. salicina***	**2 X MIC *B. salicina***
16	0	1	1	1	1
14.4	1.6	0.55	0.275	0.275	0.0687
12.8	3.2	0.6	0.075	0.3	0.15
11.2	4.8	0.325	0.325	0.325	0.0812
9.6	6.4	0.35	0.35	0.087	0.0875
8	8	0.375	0.375	0.375	0.375
6.4	9.6	0.4	0.8	1.6	0.4
4.8	11.2	0.425	0.85	0.85	0.425
3.2	12.8	0.45	0.9	0.9	0.45
1.6	14.4	0.475	0.475	0.95	0.118
0	16	1	1	1	1
	Average FIC	**0.562963**	**0.491667**	**0.629167**	**0.240**

Table G3: Sum fractional inhibitory concentrations for *O. sphaerocarpa* bark and *B. salicina* bark with different concentrations of *S. cordatum* bark, tested against *E. coli*.

Concentrations (mg/ml)		**ΣFIC**			
***O. sphaerocarpa* bark**	***B. salicina* bark**	**Without *S. cordatum***	**½ MIC *S. cordatum***	**MIC *S. cordatum***	**2 X MIC *S. cordatum***
16	0	1	1	1	1
14.4	1.6	0.45625	0.903	1.806	0.903
12.8	3.2	0.825	0.806	1.612	0.806
11.2	4.8	0.7375	1.418	0.709	1.419
9.6	6.4	0.325	1.225	1.225	1.225
8	8	1.125	0.516	2.062	1.031
6.4	9.6	0.95	0.837	1.675	1.675
4.8	11.2	1.55	0.644	2.575	0.644
3.2	12.8	1.2	0.9	3.6	1.8
1.6	14.4	0.85	0.256	1.025	0.512
0	16	1	1	1	1
	Average FIC	**0.888542**	**0.834028**	**1.810069**	**1.113**

APPENDIX H: Sum Fractional Inhibitory Concentration for Two Plant Combination Against Human Kidney Epithelial cells

Table H1: Sum fractional inhibitory concentrations for *B. salicina* bark with *S. cordatum* bark, tested against human kidney epithelial cells.

Concentrations (µg/ml)		**ΣFIC**	
***B. salicina* bark**	***S. cordatum* bark**	**Experiment 1**	**Experiment 2**
100	0	1	1
80	1	0.868	0.749
50	10	0.542	0.434
20	25	0.211	0.229
10	50	0.205	0.156
5	100	0.280	0.318
1	200	0.654	0.822
0	400	Average FIC	**0.456**
		s.d.	**0.006**

Table H2: Sum fractional inhibitory concentrations for *O. sphaerocarpa* bark with *S. cordatum* bark, tested against human kidney epithelial cells.

Concentrations (µg/ml)		**ΣFIC**	
***S. cordatum* bark**	***O. sphaerocarpa* bark**	**Experiment 1**	**Experiment 2**
100	0	1	1
80	1	0.755	0.885
50	5	0.600	0.551
20	10	1.110	0.963
10	20	1.489	1.131
5	40	3.475	2.702
1	50	2.502	1.564
0	80	Average FIC	**1.477**
		s.d.	**0.251**

Table H3: Sum fractional inhibitory concentrations for *O. sphaerocarpa* bark with *B. salicina* bark, tested against human kidney epithelial cells.

Concentrations (µg/ml)		**ΣFIC**	
***B. salicina* bark**	***O. sphaerocarpa* bark**	**Experiment 1**	**Experiment 2**
400	0		
200	1	0.497	0.909
100	5	0.624	1.389
50	10	1.042	0.675
25	20	2.039	0.839
10	40	1.866	0.506
1	50	1.213	0.668
0	80	Average FIC	**1.022**
		s.d.	**0.271**

University
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Johannesburg

Human Research Ethics Committee (Medical)
(formerly Committee for Research on Human Subjects (Medical)

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Ref: W-CJ-090424-10
29/04/2009

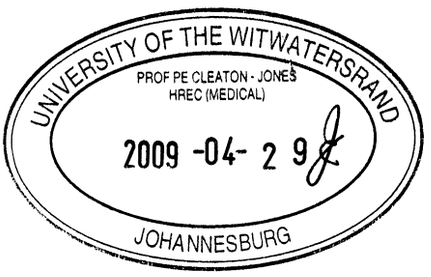
TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator: **Dr R L van Zyl & Ms G F Sibandze (Student no 0618480K)**

Project title: **Pharmacological properties of some Swazi medicinal plants.**

Reason: This is a wholly laboratory study using commercial cell lines - Graham. There are no humans involved.



Professor Peter Cleaton-Jones
Chair: Human Research Ethics Committee (Medical)

copy: Anisa Keshav, Research Office, Senate House, Wits